Distinct pattern of oxidative DNA damage and DNA repair in follicular thyroid tumours

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Abstract

Increased oxidative stress has been linked to thyroid carcinogenesis. In this paper, we investigate whether oxidative DNA damage and DNA repair differ in follicular adenoma (FA) and follicular thyroid carcinoma (FTC). 7,8-Dihydro-8-oxoguanine (8-OxoG) formation was analysed by immunohistochemistry in 46 FAs, 52 FTCs and 18 normal thyroid tissues (NTs). mRNA expression of DNA repair genes OGG1, Mut Y homologue (MUTYH) and endonuclease III (NTHL1) was analysed by real-time PCR in 19 FAs, 25 FTCs and 19 NTs. Induction and repair of oxidative DNA damage were studied in rat FRTL-5 cells after u.v. irradiation. Moreover, activation of DNA damage checkpoints (ataxia telangiectasia mutated (ATM) and H2A histone family, member X (H2AFX (H2AFX))) and proliferation index (MIB-1) were quantified in 28 non-oxyphilic and 24 oxyphilic FTCs. Increased nuclear and cytosolic 8-OxoG formation was detected in FTC compared with follicular adenoma, whereby cytosolic 8-OxoG formation was found to reflect RNA oxidation. Significant downregulation of DNA repair enzymes was detected in FTC compared with FA. In vitro experiments mirrored the findings in FTC with oxidative stress-induced DNA checkpoint activation and downregulation of OGG1, MUTYH and NTHL1 in FRTL-5 cells, an effect that, however, was reversible after 24 h. Further analysis of FTC variants showed decreased oxidative DNA damage, sustained checkpoint activation and decreased proliferation in oxyphilic vs non-oxyphilic FTC. Our data suggest a pathophysiological scenario of accumulating unrepaired DNA/RNA damage in FTC vs counterbalanced DNA/RNA damage and repair in FA. Furthermore, this study provides the first evidence for differences in oxidative stress defence in FTC variants with possible implications for therapeutic response and prognostic outcome.

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Introduction

The thyroid follicular cell is at increased risk of oxidative stress resulting in oxidative DNA damage induced by reactive oxidative species (ROS). In particular, an imbalance between pro- and antioxidative factors has been suggested as an important mechanism in thyroid tumourigenesis (Krohn et al. 2005, Song et al. 2007). In thyrocytes, H₂O₂ is generated by thyroid oxidases in response to thyrotopin and acts as an essential cofactor for thyroid peroxidase during thyroid hormone synthesis (De Deken et al. 2002, Song et al. 2010). Excess of H₂O₂ is eliminated by the antioxidative system, e.g. superoxide dismutase, glutathione peroxidases and peroxiredoxins (Köhrle et al. 2005, Song et al. 2007). Using a rodent model, Maier et al. (2006) have recently demonstrated that, in contrast to other organs, normal thyroid tissue (NT) exhibits an eight to ten times higher rate of spontaneous mutations. Furthermore, in a very recent study, Driessens et al. (2009) have shown that H₂O₂ causes single- and double-strand DNA breaks in thyroid cells. This in vitro evidence of a particularly mutagenic environment of the thyroid is in good agreement with the clinical picture of nodular thyroid disease: i) the very high prevalence of nodular thyroid lesions in up to 50% of the adult population, of which a substantial portion is clonal (Volzke et al. 2003, Krohn et al. 2005), ii) the frequent finding of incidental papillary microcarcinoma, which arises from somatic mutations in the RAS–BRAF–MAPK cascade in operated thyroids (Carlini et al. 2005, Shattuck et al. 2005, Kondo et al. 2006) and iii) the increasing prevalence of thyroid cancer with age, with a peak in the sixth and seventh decade of life in women and men respectively (Liu et al. 2001, Husmann et al. 2010, Dal Maso et al. 2011).

ROS-induced DNA damage involves single- or double-strand DNA breaks, purine and pyrimidine or deoxyribose modifications as well as DNA cross links (Hoeijmakers 2009, Jackson & Bartek 2009, van Loon et al. 2010). The most widely studied DNA oxidation product is 7,8-dihydro-8-oxoguanine (8-OxoG),
a highly mutagenic product of the interaction of ROS and DNA (Beckmann & Ames 1997, Jackson & Bartek 2009, van Loon et al. 2010). If unrepaird, the base lesion promotes a G→C to T→A transition following replication (Kreutzer & Essigmann 1998). Detection and repair of oxidative DNA damage are conducted by the base excision repair (BER) system (Klugland et al. 1999, Minowa et al. 2000, David et al. 2007, Russo et al. 2007). In eukaryotes, in particular, the 8-OxoG DNA glycosylase 1 (OGG1) plays a prominent role in recognition and excision of 8-OxoG sites (Rosenquist et al. 2007). In addition, Mut Y homologue (MUTYH) and endonuclease III (NTHL1) are encoding BER enzymes responsible for repair of oxidative purine and pyrimidine DNA base lesions respectively (Al-Tassan et al. 2002, Russo et al. 2007).

So far, no systematic study has been performed to assess the extent of oxidative DNA damage in thyroid tumours. In this paper, we explore the hypothesis that increased oxidative DNA damage may be present with ongoing thyroid transformation leading to genomic instability and dedifferentiation. In this paper, we studied the extent of 8-OxoG formation and oxidative DNA damage repair in follicular adenomas (FAs) and follicular thyroid carcinomas (FTCs) in order to get further information on whether follicular thyroid tumours evolve in a sequential (adenoma–carcinoma sequence) or distinct way. Furthermore, we applied an in vitro model of the oxidative stress response to compare the physiological response with the in vivo situation we observed in follicular thyroid tumours. We found that i) the formation of 8-OxoG DNA adducts is increased in FTC and FA compared with NT, ii) this is coincided by the decreased gene expression of BER enzymes in FTC and iii) the induction of the DNA damage response in FTC is downregulated compared with FA. In addition, we found discrepancies in the extent of DNA damage in oxyphilic FTC compared with non-oxyphilic FTC variants.

Materials and methods

Thyroid samples

Thyroid samples were obtained from patients undergoing thyroid surgery for nodular thyroid disease or thyroid cancer. Thyroid specimens were obtained from the Department of Surgery, Martin-Luther-University Hospital, Halle, and from the Department of Pathology and Neuropathology, University of Duisburg-Essen, Essen, Germany.

Duplicates of 19 hypofunctional FAs, 19 corresponding NTs from the same patient and 25 FTCs were studied for mRNA expression of hOGG1, hMUTYH and hNTHL1. For immunohistochemical analysis of 8-OxoG formation, DNA damage response (ataxia telangietasia mutated (ATM) and H2A histone family, member X (H2AFX) and proliferation (Ki-67 protein expression), paraffin-embedded tissue sections from 46 FAs, 52 FTCs (comprising 28 non-oxyphilic and 24 oxyphilic) and 18 NTs were studied.

For exclusion of an age-related influence of oxidative stress on our data, FTC and FA patients were age matched. The median age of FTC patients was 55 (mean 51.08 ± 16.29) years and of FA patients 52 (mean 52.39 ± 15.15) years.

Classification of the thyroid nodules was performed by the pathologists according to World Health Organization (WHO) criteria. The term follicular adenoma was applied for benign follicular lesions, which were completely encapsulated. The term follicular carcinoma was used if capsule invasion and/or vascular invasion were present. The term oxyphilic tumour (syn. oncocytic, Hürthle cell tumour) was applied in case of a tumour with abundant granular eosinophilic cytoplasm, as a consequence of increased mitochondrial content (Vonate et al. 2002, Porcelli et al. 2010). All patients gave informed consent and the study was approved by the local ethics committee.

Cell culture

Follicular rat thyroid (FRTL-5) cells (kindly provided by Prof. Di Lauro, Naples) were split into 6-well plates (3 × 10⁵ cells/well) and were cultured in a 2:1:1 mixture of DMEM:Ham’s F12:MCDB104 supplemented with 5% newborn calf serum (all from Gibco BRL, Life Technologies), 10 mg/ml insulin, 0.4 mg/ml hydrocortisone (Calbiochem, San Diego, CA, USA), 45 mg/ml ascorbic acid (Sigma), 5 mg/ml transferrin (Calbiochem) and 5 mU/ml bovine TSH (Sigma) until 70% confluence. Cells were exposed to a single dose of u.v. irradiation (0-001 J/cm²) at room temperature. Four, 8 and 24 h after u.v. irradiation, FRTL-5 cells were washed twice with PBS and were shock frozen in liquid nitrogen and stored at −80°C. Controls were treated identically except for u.v. irradiation. The experiment was performed in triplicates.

RNA extraction and real-time RT-PCR

RNA was isolated from snap-frozen thyroid tissue and FRTL-5 cells using TRIzol reagent (Invitrogen). One microgram of RNA per sample was reverse transcribed in a final mixture of 5× first-strand buffer (250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM MgCl₂ (Gibco BRL), 0.5 mM dNTPs, 5 mM dithiothreitol (Gibco BRL), 15 U Prime RNAse inhibitor (PeqLab, Erlangen, Germany), 0.5 mg random hexamer primers and 200 U Moloney murine leukemia virus reverse
transcriptase (Gibco BRL). Reverse transcription was performed at 37 °C for 60 min and 94 °C for 5 min. Quantitative real-time PCR using exon spanning primer for human and rat OGG1, MUTYH and NTHL1 was performed using the LightCycler System and LightCycler–DNA Master SYBR Green I kit (Roche) according to the manufacturer’s instructions. Annealing temperatures and MgCl2 concentrations were optimised to create a one-peak melting curve. Primers and PCR conditions were as follows:

**Primer sequences for human tissue:**

**OGG1** Forward: 5'-GAA ATT CCA AGG TGT GCG AC-3'  
Reverse: 5'-CCA TG A CA TCA TCA AGC TG-3'

**MUTYH** Forward: 5'-GAG GAG TGT GCT CCC AAC AC-3'  
Reverse: 5'-AGG CTG TT CAG AAG AGA GG-3'

**NTHL1** Forward: 5'-CGG AAA GCA CAG AGA CTG C-3'  
Reverse: 5'-TGG AGA GCA TCA GTG ACA GC-3'

**PCR conditions:**

After initial denaturation (30 s) at 95 °C, PCR was carried out for 40 cycles: for OGG1, 95 °C for 0 s, 72 °C for 7 s and 59 °C for 8 s, 4 mM MgCl2; for MUTYH, 95 °C for 0 s, 72 °C for 7 s and 60 °C for 8 s, 4 mM MgCl2; and for NTHL1, 95 °C for 0 s, 72 °C for 7 s and 60 °C for 11 s, 3 mM MgCl2. The LightCycler Software calculated the threshold cycles.

**Primer sequences for rat FRTL-5 cells:**

**Ogg1** Forward: 5'-CAG CTC TAT AGG CAC TGG GC-3'  
Reverse: 5'-CTA CTG CTG GAC CAG CCA GG-3'

**Myh** Forward: 5'-CAA CCA CAA GAG GAG GGG AA-3'  
Reverse: 5'-CTG CAT AGG CCT TTC TCT CG-3'

**Nth** Forward: 5'-GTG CAT GTC GAG CAA GTG AT-3'  
Reverse: 5'-CAT TCT TTT GAG GGG TTC ATG AT-3'

**PCR conditions:**

After initial denaturation (30 s) at 95 °C, PCR was carried out for 40 cycles: for Ogg1, 95 °C for 0 s, 72 °C for 7 s and 62 °C for 12 s, 3 mM MgCl2; for Myh, 95 °C for 0 s, 72 °C for 7 s and 58 °C for 12 s, 3 mM MgCl2; and for Nth, 95 °C for 0 s, 72 °C for 7 s and 62 °C for 13 s, 3 mM MgCl2. The LightCycler Software calculated the threshold cycles.

The mRNA expression of the housekeeping protein β-actin and ribosomal rS6 protein was used for normalisation of mRNA expression in human samples and FRTL-5 cells respectively. To quantify changes in mRNA expression patterns, real-time PCR was performed using the LightCycler DNA Master SYBR Green I kit (Roche) as described previously (Krause et al. 2008, Karger et al. 2009). In the thyroid tissues, the fold difference (n) in up- or downregulation of mRNA expression was calculated based on Pfaffl’s methodology (Pfaffl 2001) as follows:

\[
\frac{2^{\Delta \Delta Ct} \text{threshold cycle of control cells} - \text{threshold cycle of u.v.-treated cells}}{2^{\Delta \Delta Ct} \text{threshold cycle of NT} - \text{threshold cycle of diseased tissues (DT)}}
\]

‘Diseased tissue’ (DT) corresponds to FA, and FTC, whereas ‘normal tissue’ corresponds to the surrounding tissue of FA. In the FRTL-5 cells, fold difference (n) in up- or downregulation of mRNA expression was calculated (Pfaffl 2001) as follows:

\[
\frac{2^{\Delta \Delta Ct} \text{threshold cycle of normal cells} - \text{threshold cycle of treated cells}}{2^{\Delta \Delta Ct} \text{threshold cycle of control cells} - \text{threshold cycle of u.v.-treated cells}}
\]

The Mann–Whitney U test within the SPSS 11.5 Software (SPSS Inc Chicago, IL, USA) was applied to calculate the statistical significance of differences in the mRNA expression of the respective genes between thyroid tissues.

**Western blot analysis**

Western blot analysis was performed using antibodies against H2AFX (Cell Signaling, Charlottesville, VA, USA) and the S139-phosphorylated form of H2AFX (γH2AFX; S139-H2AFX; Upstate Biotechnology, Lake Placid, NY, USA). Cells were lysed in buffer containing 10 mM Tris, 400 mM NaCl, 1 mM EDTA and 0.1% NP-40 (all from Sigma). Fifty micrograms of protein were separated on 14% SDS gels followed by semi-dry western blotting. Subsequently, membranes were blocked with 5% BSA in TBST (50 mM Tris–HCl (pH 7.5) and 150 mM NaCl containing 0.05% Tween-20). Blots were probed overnight with the following dilutions of antibodies in TBST containing 5% BSA: anti-H2AFX (1:1000), anti-p-S139-H2AFX (γH2AFX; 1:1000) and anti-β-actin (1:500). After incubation with a secondary anti-rabbit antibody coupled to HRP (Cell Signaling), immunocomplexes were visualised by enhanced chemiluminescence (Pierce, Rockford, IL, USA). β-Actin was used as a loading control.

**Immunohistochemistry**

Paraffin-embedded tissue sections (2 μm) were incubated with one of the following antibodies: anti-8-OHdG/8-OHG, 1:500 (Rockland, Gilbertsville, PA, USA); anti-phospho-S1981-ATM, 1:200 (Abcam); anti-phospho-S139-H2AFX, 1:200 (Cell Signaling) as well as anti-Ki-67 (MIB-1) antibody, 1:100 (DAKO, Glostrup, Denmark) using previously described immunohistochemistry protocols (Krause et al. 2007, Karger et al. 2009).

For RNA elimination before incubation with the anti-8-OHdG/8-OHG antibody, tissue sections were
pretreated with 7000 U/ml RNAse A (Qiagen Sciences) for 12 h at 37 °C.

Immunoreactivity was demonstrated using a biotinylated secondary anti-rabbit antibody, streptavidin peroxidase and diaminobenzidine. Sections were counterstained with hemalaun and mounted in Aquatex (Merck).

H₂O₂ pretreated NT sections (for 8-OxoG staining) and tissue sections from small intestine and colon cancer (for p-ATM, γH2AFX and MIB-1 staining) were used as positive controls.

Triplicates per tissue sample were investigated, i.e. three sequential sections. The overall amount of nuclear and cytosolic 8-OxoG immunoreactivity in thyrocytes was determined in ten randomly selected sections at 200× magnification per slide. Subsequently, the mean ± S.D. and the median of the percentage of positively stained nuclei and cytoplasm/thyrocytes were calculated for FA, FTC and separately for the FTC subtypes. The nuclear and cytosolic 8-OxoG immunoreactivity was categorised according to Bartkova et al. (2005): i) negative (no positive staining or up to 1% of positive cells), ii) low (with at least 20% of the section showing 2–35% positive cells), iii) moderate (with at least 20% of the section showing 36–75% positive cells) and iv) strong (with at least 20% of the section showing 76–100% positive cells). Determination of the index of proliferation (positive nuclear Ki-67/MIB-1 labelling) was performed by counting 500 tumour cells in the most intense staining areas with 400× magnification and was quantified as percentage. The Mann–Whitney U test within the SPSS 11.5 Software was applied to calculate the statistical significance of differences in protein expression.

Results

FTC shows accumulation of unrepaired DNA adducts with increased formation of 8-OxoG adducts

To study the extent of oxidative DNA damage in follicular thyroid tumours, we used a monoclonal antibody, which detects 8-OHdG/8-OHG oxidation products induced by ROS. Significantly increased nuclear and cytosolic 8-OxoG immunoreactivity was found in FTCs and FAs compared with NTs (Fig. 1A). The mean nuclear 8-OxoG staining was 64.2±29.1% in FA (median 70%) and 64.7±33.1% in FTC (median 85%, not significant). According to the modified scoring model of Bartkova et al. (2005), 47.6% of FAs showed a strong nuclear 8-OxoG staining vs 55.3% of FTCs (strong = at least 20% of the section showing 76–100% nuclei with 8-OxoG immunoreactivity). On the subcellular level, 8-OxoG staining was more pronounced in the cytosol than in the nucleus both in FTC and in FA. Thus, the mean cytosolic 8-OxoG staining in FA was 70.6±28.2% (median 85%) and in FTC 81.5±27.8% (median 100%; Fig. 1B). This difference was significant at P<0.02. According to the modified scoring model of Bartkova et al. (2005), 57.1% of FAs showed a strong overall cytosolic 8-OxoG staining vs 72.3% of FTCs. The results of the scoring analysis of nuclear and cytosolic 8-OxoG staining in FA and FTC are shown in Supplementary Figure 1, see section on supplementary data given at the end of this article. No correlation was found between the amount/strength of nuclear and cytosolic 8-OxoG expression and the respective FTC tumour stages (Table 1).

Recent studies indicate that RNA, such as mRNA and rRNA, is very vulnerable to oxidative damage (Kong & Lin 2010). To clarify whether cytosolic 8-OxoG formation in FTC and FA corresponds to oxidative mitochondrial DNA or mRNA/rRNA damage, we subjected tissue samples to RNAse treatment before 8-OxoG staining. We observed a marked decrease in cytosolic 8-OxoG immunoreactivity, which suggests that this cytosolic 8-OxoG staining reflects oxidative mRNA/rRNA damage (Fig. 2).

Figure 1 (A) Representative images of 8-OxoG immunohistochemistry in benign and malignant thyroid follicular tumours and normal thyroid tissue: FTC displays higher median nuclear 8-OxoG staining and significantly higher median cytosolic 8-OxoG staining than FA. Nuclear and cytosolic 8-OxoG accumulation is much lower in NT. Magnification: 400×. (B) Box plots show median and distribution (box area = 50% of samples) of the percentage of 8-OxoG positively stained thyrocytes (nuclear and cytosolic) in FA and FTC. The mean overall amount of nuclear 8-OxoG staining was 64.2±29.1% in FA (median 70%) and 64.7±33.1% in FTC (median 85%). The mean overall amount of cytosolic 8-OxoG staining in FA was 70.6±28.2% (median 85%) and in FTC 81.5±27.8% (median 100%; B). This difference was calculated as significant (P<0.02; Mann–Whitney U test). FA, follicular adenoma; FTC, follicular carcinoma; NT, normal thyroid tissue. Full colour version of this figure available via http://dx.doi.org/10.1530/JME-11-0119.
DNA damage repair genes are downregulated in FTCs

As detection and repair of oxidative DNA damage are conducted by the BER system, we studied the gene expression of OGG1, MUTYH and NTHLI in FTC, FA and NT. mRNA expression of the three BER genes was detected in all thyroid tissue samples. Compared with FA, FTC showed a significant downregulation of all three BER genes in FTC (P<0.009 for OGG1, P<0.02 for MUTYH and P<0.023 for NTHLI; Fig. 3). The comparison of FA and NT showed increased mRNA expression of all three BER genes in the follicular adenomas, but this failed to reach statistical significance (Fig. 3). Additional investigations on the correlation between the gene expression levels of OGG1, NTHLI and MUTYH and the respective tumour stages of all FTC yielded no positive results. All data are shown in Table 2.

Induction of oxidative stress in FRTL-5 cells mirrors in vivo finding in FTC

To study the dynamics of oxidative DNA damage and repair in thyrocytes, we used u.v. irradiation of FRTL-5 cells as an in vitro model for oxidative stress induction. U.v. irradiation of FRTL-5 cells led to a significant mRNA downregulation of all three investigated BER genes after 4–8 h (P<0.025 for Ogg1, P<0.017 for Mutyh and P<0.025 for Nthli; Fig. 4A) in comparison with the non-irradiated control FRTL-5 cells. After 24 h, the mRNA expression of all three BER genes was restored to the level of control cells (Fig. 4A).

In addition, we investigated the activation of the DNA damage response by the phosphorylation of histone H2AFX. Upon DNA damage, activated histone H2AFX is thought to restructure chromatin and assist in the recruitment of DNA repair and signalling factors (van Attikum & Gasser 2009). Before u.v. irradiation, we observed only faint H2AFX phosphorylation and absence of γH2AFX. Four and 8 h after irradiation, we found a strong induction of γH2AFX expression (Fig. 4B). Thereby, the kinetics of H2AFX protein activation and mRNA expression of BER genes were reciprocal.

Differences in 8-OxoG formation and DNA damage response between oxyphilic and non-oxyphilic FTC variants

While 8-OxoG formation was increased in FTC compared with FA (Fig. 1), we particularly noted differences in 8-OxoG-staining intensity depending on the histopathological subtype of FTC investigated. We thus decided to perform subgroup analysis of our FTC samples comprising oxyphilic FTC and non-oxyphilic FTC. We found that 8-OxoG staining was more pronounced in non-oxyphilic FTC vs oxyphilic FTC with significant differences in the degree of nuclear 8-OxoG formation between the two FTC variants (non-oxyphilic FTC, mean 75.4±30.7%, median

Table 1 Correlation of histological subtype, patient’s gender, age and Tumour Node metastasis (TNM) staging with nuclear and cytosolic 8-OxoG expression of n=47 follicular thyroid carcinomas investigated in the study

<table>
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<tr>
<th>No.</th>
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<th>Age, gender</th>
<th>TNM</th>
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<th>8-Oxo cytosolic</th>
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*No. in tissue array; †Year of collection; NOF, Non-oxyphilic FTC; OF, Oxyphilic FTC; HS, Histological subtype.
85% positive nuclei vs oxyphilic FTC, mean 53.5 ± 31.8%, median 65% positive nuclei; P<0.015; Fig. 5A and B). Whereas the mean nuclear 8-OxoG staining in FA (64.2 ± 29.1%; median 70%) and in the whole group of FTC (64.7 ± 33.1%; median 85%) was not significantly different, the comparison of 8-OxoG formation between non-oxyphilic FTC vs FA showed a clear preponderance of nuclear 8-OxoG accumulation in non-oxyphilic FTC (mean 75.4 ± 30.7% (median 85%) vs mean 64.2 ± 29.1% (median 70%)). This difference was significant at P<0.04. Comparison of cytosolic 8-OxoG staining in non-oxyphilic FTC vs FA (mean 85.4 ± 24.6% (median 100%) vs mean 70.6 ± 28.2% (median 85%)) and the whole group of FTC vs FA showed significant (P<0.007 and P<0.02 respectively) expression differences. Interestingly, non-oxyphilic FTC also exhibited a higher rate of cellular proliferation reflected by the MIB-1 index of proliferation (median 2:1%) compared with oxyphilic FTC (median 1:0%); Fig. 5C).

To clarify whether the differences in 8-OxoG formation in the FTC variants correlate with different degrees of activation of cell cycle checkpoint kinases, we performed immunohistochemistry using specific antibodies against the activated form of ATM (p-S1981) and H2AFX (p-S139) (Bartkova et al. 2005). Phosphorylation of these markers in response to DNA damage induces DNA repair and elicits growth arrest or apoptosis (Tanaka et al. 2007).

A moderate nuclear and low cytosolic phospho-ATM expression was detected in non-oxyphilic FTC variants, whereas a strong nuclear and cytosolic phospho-ATM expression was found in the oxyphilic tumours (Supplementary Figure 2, see section on supplementary data given at the end of this article). Furthermore, a moderate nuclear staining of γH2AFX was detected in both non-oxyphilic and oxyphilic FTCs. Additionally, in oxyphilic FTC, we observed a moderate cytosolic γH2AFX immunostaining pattern, which was completely absent in non-oxyphilic FTC (Supplementary Figure 2, see section on supplementary data given at the end of this article).

**Discussion**

In this paper, we follow the hypothesis that oxidative DNA damage is implicated in thyroid tumourigenesis and plays an important pathogenic role in follicular thyroid carcinogenesis. In our study, we concentrated on follicular tumours because their molecular pathogenesis is yet unresolved and it is not known whether follicular adenoma and carcinoma evolve in a sequential way. To explore our paradigm that increasing oxidative stress is relevant to carcinogenesis, we investigated a series of FAs and FTCs using 8-OxoG staining as an indicator of oxidative DNA damage. Strong 8-OxoG staining was observed in the nuclear and cytosolic compartments in FA and FTC, with a significantly higher cytosolic staining index in the FTC compared with FA. Further studies suggested that cytosolic 8-OxoG staining in the tumours may reflect oxidative RNA modifications.

![Figure 2](Image) **Figure 2** RNAse treatment leads to loss of cytosolic 8-OxoG staining suggesting that cytosolic staining is due to oxidative RNA damage. Representative images of cytosolic 8-OxoG immunostaining in FTC treated with 7000 U/ml RNAse for 12 h at 37 °C. Control slides were treated with buffer only. Magnification: 400 ×.

![Figure 3](Image) **Figure 3** mRNA expression of the DNA base excision repair enzymes Ogg1, Nthl1 and Mutyh in normal thyroid tissues (NTs), follicular adenoma (FA) and follicular thyroid carcinoma (FTC). Gene expression was normalised for ACTB expression and determined by real-time PCR as described in the Materials and methods section. Box plots show median and distribution (box area = 50% of samples) of OGG1, NTHL1 and MUTYH mRNA expression in the explored thyroid tissues, which allowed statistically significant separation: P<0.009 (A), P<0.02 (B), P<0.023 (C) and P<0.04 (D; Mann–Whitney U test). (A) OGG1 FA vs FTC; (B) NTHL1 FA vs FTC; (C) MUTYH FA vs FTC and (D) OGG1 NT vs FTC.
As oxidative DNA damage may be due to i) increased oxidative stress and/or ii) impaired DNA repair, the gene expression of DNA damage repair enzymes *OGG1*, *MUTYH* and *NTHL1* was studied. Interestingly, all three BER genes were significantly downregulated in FTC but were upregulated in FA compared with NT.

To investigate whether these changes indeed reflect DNA damage response, we exposed FRTL-5 cells to u.v. irradiation, which is an established mode for induction of oxidative stress (Driessens et al. 2009). U.v. irradiation resulted in increased activation of H2AFX and was accompanied by mRNA downregulation of the DNA damage repair genes *Ogg1*, *Mutyh* and *Nthl1* in FRTL-5 cells. Thus, these findings mirror the *in vivo* picture in FTC, which likewise display activation of cell cycle checkpoints in contrast to follicular adenoma (Krause et al. 2011). However, in FRTL-5 cells, the u.v.-induced changes were temporary, reflecting the physiological response in a ‘healthy’ system, while changes in FTC are not. This is in line with FTC representing a ‘diseased’ system hallmarked by constitutive activation of specific signal transduction (Fagin 2002, Kondo et al. 2006) promoting, e.g. oxidative stress and/or impairing DNA repair.

During analysis of our FTC samples, we noted differences in the extent of 8-OxoG formation depending on their morphological appearance. In a subsequent analysis, we addressed this issue more specifically for the two FTC variants (oxyphilic and non-oxyphilic) under study. Oxyphilic follicular carcinomas are largely composed of oncocytic cells (>75% of the tumour cells according to WHO criteria), which contain large masses of mitochondria and are genetically characterised by mitochondrial DNA mutations in contrast to non-oxyphilic FTC, which display constitutive activation of PI3K activation (Hu et al. 2008, Gasparre et al. 2010). Two interesting pictures emerged in our subset analysis. First, oxyphilic FTC displayed significantly lower nuclear and cytosolic 8-OxoG...
staining than non-oxyphilic FTC. Second, activation of DNA damage response markers was more pronounced in oxyphilic compared with non-oxyphilic FTC, whereby the cytosolic finding of activated ATM and H2AFX in oxyphilic FTC most likely reflects mitochondrial DNA alterations in line with the characteristic finding of mtDNA mutations (Gasparre et al. 2010). However, the differences in nuclear 8-OxoG formation and nuclear DNA damage response between the two FTC variants could argue for preservation of a more sustained cell cycle control in oxyphilic FTC, whereby checkpoint activation allows repair of oxidative DNA damage resulting in a lower degree of persistent DNA damage. As these studies were performed on archival paraffin-embedded tissues, we were not able to study mRNA expression of DNA repair genes. However, our rationale is substantiated by the finding of a much lower (but below statistical significance) proliferation rate in oxyphilic compared with non-oxyphilic FTC, which has also been previously described by other investigators (Vonate et al. 2002).

Though the biological behaviour of oxyphilic tumours is still debated, our findings of increased (nuclear) checkpoint activation, decreased proliferation and decreased (nuclear) oxidative damage in oxyphilic FTC would suggest a more benign course of disease. Very recent evidence for this reasoning comes from a study by Porcelli et al. (2010) who showed that the occurrence of disruptive mitochondrial DNA mutations mostly in respiratory complex I, which are the genetic hallmarks of oncocyctic cell transformation, contribute to contain the tumour in a benign state by inhibiting stabilisation of the hypoxia-inducible factor 1α.

We believe that clinical studies addressing this issue more specifically are required and are mandatory to interpret the success of yet experimental therapies, e.g. with tyrosine kinase inhibitors or other drugs targeting oxidative stress response in patients with different FTC variants.

In summary, we provide the first evidence that increased oxidative stress and stress-induced DNA damage are the hallmarks of follicular thyroid tumours with downregulation of the DNA repair machinery in FTC vs upregulation in benign FA. Furthermore, we demonstrate that oxidative damage is more pronounced in non-oxyphilic compared with oxyphilic FTC. In our view, this finding merits further investigation as to its biological outcome and clinical relevance.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JME-11-0119.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

S K and K K planned and carried out the experiments, C E and C W helped with experiments and analysed data. O G and H D were the surgeons who removed the thyroid tumours; S Y, S G and K W S were the pathologists who carried out histological classification of the thyroid tumours. D F planned the experiments and supervised all the work on this paper. All authors were involved in writing the paper and had final approval of the submitted and published versions.

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