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 thyrotropin 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 unrepaired, the base lesion promotes a G→C to T→A transversion following replication (Kreutzer & Essigmann 1998). Detection and repair of oxidative DNA damage are conducted by the base excision repair (BER) system (Klungland *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.* 1997). 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 telangiectasia 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. oncocyctic, 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 (3×10^5 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/cm^2) 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 $5 \times$ first-strand buffer (250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl_2 (Gibco BRL), 0.5 mM dNTPs, 5 mM dithiothreitol (Gibco BRL), 15 U Prime RNase inhibitor (PqLab, Erlangen, Germany), 0.5 mg random hexamer primers and 200 U Moloney murine leukaemia 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:

<i>OGG1</i>	Forward: 5'-GAA ATT CCA AGG TGT GCG AC-3' Reverse: 5'-CCA TTG ACA TCA TCA AGC TG-3'
<i>MUTYH</i>	Forward: 5'-GAG GAG TGT GCT CCC AAC AC-3' Reverse: 5'-AGG CTG TTC CAG AAC ACA GG-3'
<i>NTHL1</i>	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:

<i>Ogg1</i>	Forward: 5'-CAG CTC TAT AGC CAC TGG GC-3' Reverse: 5'-CTA CTT CTG GAC CAG CCA GG-3'
<i>Myh</i>	Forward: 5'-CAA CCA CAA GAG AAG GGG AA-3' Reverse: 5'-CTG CAT AGG CTC TCC TGT CC-3'
<i>Nth</i>	Forward: 5'-GTG CAC GTT GAG CAA GTG AT-3' Reverse: 5'-CAC TTT GAG GGG TTC AGC 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:

$$n = \frac{2^{(\text{threshold cycle of NT} - \text{threshold cycle of diseased tissues (DT)})}}{2^{(\text{tc ACTB (NT)} - \text{tc ACTB (DT)})}}.$$

'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:

$$n = \frac{2^{(\text{threshold cycle of control cells} - \text{threshold cycle of u.v.-treated cells})}}{2^{(\text{tc rS6 (control)} - \text{tc rS6 (u.v.-treated cells)})}}.$$

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 $64.2 \pm 29.1\%$ in FA (median 70%) and $64.7 \pm 33.1\%$ 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 $70.6 \pm 28.2\%$ (median 85%) and in FTC $81.5 \pm 27.8\%$ (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 $64.2 \pm 29.1\%$ in FA (median 70%) and $64.7 \pm 33.1\%$ in FTC (median 85%). The mean overall amount of cytosolic 8-OxoG staining in FA was $70.6 \pm 28.2\%$ (median 85%) and in FTC $81.5 \pm 27.8\%$ (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

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

No. ^a	HS	Year ^b	Age, gender	TNM	8-Oxo nuclear	8-Oxo cytosolic
1	NOF	1999	29, M	pT3 Nx Mx	85	100
2	NOF	1999	26, F	p2T Nx Mx	35	35
3	OF	1999	62, M	p2T Nx Mx	10	10
4	NOF	1999	23, F	p2T Nx Mx	85	100
5	NOF	2000	41, F	p2T Nx Mx	85	100
6	NOF	2000	57, F	p2T Nx Mx	100	100
7	NOF	2000	36, F	p2T Nx Mx	100	100
8	OF	2000	65, F	p2T Nx Mx	85	100
9	OF	2000	43, F	p2T Nx Mx	20	20
10	NOF	2000	31, F	p2T Nx Mx	100	100
11	OF	2000	59, F	p2T Nx Mx	—	—
12	OF	2000	24, M	p2T Nx Mx	20	10
13	OF	2000	70, F	p2T Nx Mx	20	100
14	NOF	2000	61, F	p2T Nx Mx	85	85
15	NOF	2000	35, M	p2T Nx Mx	100	65
16	OF	2000	48, M	p3T Nx Mx	85	100
17	NOF	2000	61, F	p2T Nx Mx	—	—
18	NOF	2000	77, M	p2T Nx Mx	85	65
19	NOF	2000	31, F	p2T Nx Mx	—	—
20	NOF	2001	64, M	p2T Nx Mx	100	100
21	NOF	2005	61, F	p2T Nx Mx	100	100
22	OF	2005	61, F	p2T Nx Mx	85	100
23	OF	2005	73, F	p2T Nx Mx	35	100
24	NOF	2005	61, F	p2T Nx Mx	100	85
25	NOF	2005	50, F	p2T Nx Mx	85	100
26	OF	2005	19, F	p2T Nx Mx	65	65
27	OF	2005	51, F	p2T Nx Mx	35	35
28	OF	2005	69, F	p2T Nx Mx	100	100
29	OF	2005	83, F	p2T Nx Mx	85	100
30	NOF	2005	59, F	p4T N0 Mx	100	100
31	NOF	2005	38, F	p2T Nx Mx	—	—
32	NOF	2005	57, F	p2T Nx Mx	35	30
33	OF	2005	83, F	p2T Nx Mx	10	100
34	NOF	2005	31, F	p2T Nx Mx	—	—
35	OF	2005	64, F	p2T Nx Mx	5	100
36	OF	2005	44, F	p2T Nx Mx	10	65
37	NOF	2005	49, F	p2T Nx Mx	100	100
38	OF	2005	49, F	p2T Nx Mx	90	85
39	NOF	2005	48, M	p2T Nx Mx	5	100
40	OF	2005	56, F	p2T Nx Mx	65	100
41	NOF	2005	49, M	p2T Nx Mx	85	100
42	NOF	2005	36, F	p2T Nx Mx	20	20
43	NOF	2005	30, F	p2T Nx Mx	85	100
44	OF	2006	65, M	p2T Nx Mx	85	85
45	OF	2006	72, M	p2T Nx Mx	35	100
46	OF	2006	70, F	p2T Nx Mx	65	85
47	OF	2006	56, M	p2T Nx Mx	50	50
48	OF	2006	33, F	p2T Nx Mx	85	85
49	NOF	2006	30, M	p3T Nx Mx	65	100
50	NOF	2006	56, F	p2T Nx Mx	5	100
51	NOF	2006	56, M	p2T Nx Mx	65	65
52	NOF-M	2006	54, M	x x M1	85	85

^aNo. in tissue array; ^bYear of collection; NOF, Non-oxophilic FTC; OF, Oxophilic FTC; HS, Histological subtype.

expression of *OGG1*, *MUTYH* and *NTHL1* 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 *NTHL1*; 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*, *NTHL1* 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 *Nthl1*; 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 expression and absence of γ H2AFX. Four and 8 h after irradiation, we found a strong induction of γ H2AFX 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 oxophilic and non-oxophilic 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 oxophilic FTC and non-oxophilic FTC. We found that 8-OxoG staining was more pronounced in non-oxophilic FTC vs oxophilic FTC with significant differences in the degree of nuclear 8-OxoG formation between the two FTC variants (non-oxophilic FTC, mean $75.4 \pm 30.7\%$, median

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 $\times$. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0119>.

85% positive nuclei vs oxyphilic FTC, mean $53.5 \pm 31.8\%$, median 65% positive nuclei; $P < 0.015$; Fig. 5A and B). Whereas the mean nuclear 8-OxoG staining in FA ($64.2 \pm 29.1\%$; median 70%) and in the whole group of FTC ($64.7 \pm 33.1\%$; 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 $75.4 \pm 30.7\%$ (median 85%) vs mean $64.2 \pm 29.1\%$ (median 70%)). This difference was significant at $P < 0.04$. Comparison of cytosolic 8-OxoG staining in non-oxyphilic FTC vs FA (mean $85.4 \pm 24.6\%$ (median 100%) vs mean $70.6 \pm 28.2\%$ (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 γ H2AFX was detected in both non-oxyphilic and oxyphilic FTCs. Additionally, in oxyphilic FTC, we observed a moderate cytosolic γ H2AFX 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 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.

Table 2 Year of collection, patient's gender and age and TNM staging are directly correlated with the respective gene expression level of *OGG1*, *NTHL1* and *MUTYH* (normalised for the house-keeping gene *ACTB*) of 25 FTCs investigated as fresh frozen specimen in the study

No. ^a	Year	Age, gender	TNM	OG- G1/ ACTB	NTH- L1/ ACTB	MUTYH ACTB
1	1996	68, F	pT3 Nx Mx	0.26	0.88	0.45
2	1996	72, M	pT3 Nx Mx	0.41	1.29	0.31
3	1996	60, F	pT4 Nx M1	0.44	1.16	0.37
4	1999	60, F	pT4 pN0 Mx	0.35	0.91	0.23
5	1999	41, F	pT4b N1 M0	0.78	0.59	0.61
6	1999	35, F	pT4 pN0 M0	0.38	1.66	0.9
7	2000	61, M	pT4 Nx Mx	0.32	1.03	0.86
8	2000	64, F	pT4b pN1b Mx	0.56	4.92	0.78
9	2001	75, M	pT3 pN1 Mx	0.27	0.65	0.81
10	2001	59, M	pT3 Nx M0	1.15	0.78	0.32
11	2001	77, F	pT3 pN0 Mx	1.23	1.54	1.16
12	2001	59, M	pT3 pNx pM1pul	0.69	0.97	0.45
13	2002	54, F	pT2a pN0 pMx	2.19	10.48	9.38
14	2002	76, F	pT2 pNx pMx	0.43	0.52	0.2
15	2002	71, F	pT4 pN0 Mx	0.21	0.27	0.08
16	2002	82, F	pT4 pN0 M1	1.42	1.92	0.93
17	2003	50, M	pT3 pN0 M0	0.33	1.13	0.59
18	2003	69, F	pT4b pNx Mx	0.32	0.19	0.13
19	2003	71, F	pT4b pN0 Mx	2.57	1.34	1.4
20	2003	71, F	pT4b N1b M1c	5.28	0.53	0.52
21	2003	72, F	pT4 pNx Mx	0.54	0.34	0.18
22	2003	74, F	pT3 Nx Mx	0.31	0.35	0.13
23	2004	50, F	pT2 pN0 pM1oss	0.61	1.65	0.56
24	2004	83, F	pT2 pN0 pM1oss	0.17	0.97	0.92
25	2004	58, F	pT2 pN0 Mx	0.25	0.73	0.67

^aNo. in tissue bank.

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 oncocyctic 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

Figure 4 (A) Changes in mRNA expression of the DNA base excision repair enzymes *Ogg1*, *Nthl1*, and *Mutyh* in FRTL-5 cells 4 h (*Nthl1*: $P < 0.017$; *Mutyh* $P < 0.025$), 8 h (*Ogg1* $P < 0.025$) and 24 h after u.v. irradiation compared with non-irradiated cells. Gene expression was normalised for *rS6* mRNA expression and was determined by real-time PCR as described in the Materials and methods section. (B) Expression and activation (phosphorylation) of the cell cycle checkpoint regulator H2AFX upon u.v. irradiation (single dose of 0.001 J/cm²) in FRTL-5 cells as investigated by western blot analysis. β -Actin was used as a loading control. Both experiments (A) and (B) were performed in triplicates.

Figure 5 (A) Representative images of 8-OxoG immunoreactivity in non-oxyphilic FTC and oxyphilic FTC, which are characterised by their larger mitochondrial content and represent a frequent FTC variant. Magnification: 400 $\times$. (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 non-oxyphilic FTC and oxyphilic FTC. The mean nuclear 8-OxoG staining was $75.4 \pm 30.7\%$ in non-oxyphilic FTC (median 85%) and $53.5 \pm 31.8\%$ in oxyphilic FTC (median 65%). This difference was calculated as significant ($*P < 0.015$; Mann-Whitney U test). The mean cytosolic 8-OxoG staining in non-oxyphilic FTC was $85.4 \pm 24.6\%$ (median 100%) and in oxyphilic FTC $77.4 \pm 30.3\%$ (median 85%). (C) Ki-67 labelling index in non-oxyphilic FTC and oxyphilic FTC showed a higher rate of cellular proliferation reflected by a higher percentage of MIB-1-positive nuclei (arrows) in non-oxyphilic FTC (median 2.1%) vs oxyphilic FTC (median 1.0%). Magnification: 400 $\times$. Full colour version of this figure available via <http://dx.doi.org/10.1530/JME-11-0119>.

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 oncogenic cell transformation, contribute to contain the tumour in a benign state by inhibiting stabilisation of the hypoxia-inducible factor 1 α .

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/10.101530/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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References

- Al-Tassan N, Chmiel NH, Maynard J, Fleming N, Livingston AL, Williams GT, Hodges AK, Davies DR, David SS, Sampson JR *et al.* 2002 Inherited variants of MYH associated with somatic G:C→T:A mutations in colorectal tumors. *Nature Genetics* **30** 227–232. (doi:10.1038/ng828)
- van Attikum H & Gasser SM 2009 Crosstalk between histone modifications during the DNA damage response. *Trends in Cell Biology* **19** 207–217. (doi:10.1016/j.tcb.2009.03.001)
- Bartkova J, Horejsi Z, Koed K, Krämer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C *et al.* 2005 DNA damage response as a candidate anti cancer barrier in early human tumorigenesis. *Nature* **434** 864–870. (doi:10.1038/nature03482)
- Beckmann KB & Ames BN 1997 Oxidative decay of DNA. *Journal of Biological Chemistry* **272** 19633–19636. (doi:10.1074/jbc.272.32.19633)
- Carlini M, Giovannini C, Castaldi F, Mercadante E, Dell'Avanzato R, Zazza S, Nania A, Santeusano G, Passeri M & Di Perna P 2005 High risk for micro carcinoma in thyroid benign diseases. Incidence in a one year period of total thyroidectomies. *Journal of Experimental & Clinical Cancer Research* **24** 231–236.
- Dal Maso L, Lise M, Zambon P, Falcini F, Crocetti E, Serraino D, Cirilli C, Zanetti R, Vercelli M, Ferretti S *et al.* & for AIRTUM Working Group 2011 Incidence of thyroid cancer in Italy, 1991–2005: time trend and age-period-cohort effects. *Annals of Oncology* **22** 957–963. (doi:10.1093/annonc/mdq467)
- David SS, O'Shea VL & Kundu S 2007 Base-excision repair of oxidative DNA damage. *Nature* **447** 941–950. (doi:10.1038/nature05978)
- De Deken X, Wang D, Dumont JE & Miot F 2002 Characterization of ThOX proteins as components of the thyroid H₂O₂-generating system. *Experimental Cell Research* **15** 187–196. (doi:10.1006/excr.2001.5444)
- Driessens N, Versteyhe S, Ghaddab C, Burniat A, De Deken X, Van Sande J, Dumont JE, Miot F & Corvilain B 2009 Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ. *Endocrine-Related Cancer* **16** 845–856. (doi:10.1677/ERC-09-0020)
- Fagin JA 2002 Minireview: branched from the start-distinct oncogenic initiating events may determine tumor fate in the thyroid. *Molecular Endocrinology* **16** 903–911. (doi:10.1210/me.16.5.903)
- Gasparre G, Bonora E, Tallini G & Romeo G 2010 Molecular features of thyroid oncocytic tumors. *Molecular and Cellular Endocrinology* **28** 67–76. (doi:10.1016/j.mce.2010.02.022)
- Hoeijmakers JH 2009 DNA damage, aging, and cancer. *New England Journal of Medicine* **361** 1475–1485. (doi:10.1056/NEJMr0804615)
- Hu J, Xia X, Cheng A, Wang G, Luo X, Reed MF, Fojo T, Oetting A, Gong J & Yen PM 2008 A peptide inhibitor derived from p53PIK phosphatidylinositol 3-kinase regulatory subunit: a novel cancer therapy. *Molecular Cancer Therapeutics* **7** 3719–3728. (doi:10.1158/1535-7163.MCT-08-0499)
- Husmann G, Kaatsch P, Katalinic A, Bertz J, Haberland J, Kraywinkel K & Wolf U 2010 Cancer in Germany 2005/2006. Incidence and trends. 7th edn. Eds Robert Koch Institute and Association of Population-based Cancer Registries in Germany. Berlin.
- Jackson SP & Bartek J 2009 The DNA-damage response in human biology and disease. *Nature* **461** 1071–1078. (doi:10.1038/nature08467)
- Karger S, Weidinger C, Krause K, Sheu SY, Aigner T, Gimm O, Schmid KW, Dralle H & Fuhrer D 2009 FOXO3a: a novel player in thyroid carcinogenesis? *Endocrine-Related Cancer* **16** 189–199. (doi:10.1677/ERC-07-0283)
- Klungland A, Rosewell I, Hollenbach S, Larsen E, Daly G, Epe B, Seeberg E, Lindahl T & Barnes DE 1999 Accumulation of premutagenic DNA lesions in mice defective in removal of oxidative base damage. *PNAS* **96** 13300–13305. (doi:10.1073/pnas.96.23.13300)
- Köhrlé J, Jakob F, Contempre B & Dumont JE 2005 Selenium, the thyroid, and the endocrine system. *Endocrine Reviews* **26** 944–984. (doi:10.1210/er.2001-0034)
- Kondo T, Ezzat S & Asa SL 2006 Pathogenetic mechanisms in thyroid follicular-cell neoplasia. *Nature Reviews. Cancer* **6** 292–306. (doi:10.1038/nrc1836)
- Kong Q & Lin CL 2010 Oxidative damage to RNA: mechanisms, consequences, and diseases. *Cellular and Molecular Life Sciences* **67** 1817–1829. (doi:10.1007/s00018-010-0277-y)
- Krause K, Karger S, Schierhorn A, Poncin S, Many MC & Fuhrer D 2007 Proteomic profiling of cold thyroid nodules. *Endocrinology* **148** 1754–1763. (doi:10.1210/en.2006-0752)
- Krause K, Eszlinger M, Gimm O, Karger S, Engelhardt C, Dralle H & Fuhrer D 2008 TFF3-based candidate gene discrimination of benign and malignant thyroid tumors in a region with borderline iodine deficiency. *Journal of Clinical Endocrinology and Metabolism* **93** 1390–1393. (doi:10.1210/jc.2006-1255)
- Krause K, Prawitt S, Eszlinger M, Ihling C, Sinz A, Schierle K, Gimm O, Dralle H, Steinert F, Sheu SY *et al.* 2011 Dissecting molecular events in thyroid neoplasia provides evidence for distinct evolution of follicular thyroid adenoma and carcinoma. *American Journal of Pathology* **179** 3066–3074. (doi:10.1016/j.ajpath.2011.08.033)
- Kreutzer DA & Essigmann JM 1998 Oxidized, deaminated cytosines are a source of C → T transitions *in vivo*. *PNAS* **95** 3578–3582. (doi:10.1073/pnas.95.7.3578)
- Krohn K, Führer D, Bayer Y, Eszlinger M, Brauer V, Neumann S & Paschke R 2005 Molecular pathogenesis of euthyroid and toxic multinodular goiter. *Endocrine Reviews* **26** 504–524. (doi:10.1210/er.2004-0005)
- Liu S, Semenciw R, Ugnat AM & Mao Y 2001 Increasing thyroid cancer incidence in Canada, 1970–1996: time trends and age-period-cohort effects. *British Journal of Cancer* **85** 1335–1339. (doi:10.1054/bjoc.2001.2061)
- van Loon B, Markkanen E & Hübscher U 2010 Oxygen as a friend and enemy: how to combat the mutational potential of 8-oxo-guanine. *DNA Repair* **9** 604–616. (doi:10.1016/j.dnarep.2010.03.004)
- Maier J, van Steeg H, van Oostrom C, Karger S, Paschke R & Krohn K 2006 Deoxyribonucleic acid damage and spontaneous mutagenesis in the thyroid gland of rats and mice. *Endocrinology* **147** 3391–3397. (doi:10.1210/en.2005-1669)
- Minowa O, Arai T, Hirano M, Monden Y, Nakai S, Fukuda M, Itoh M, Takano H, Hippou Y, Aburatani H *et al.* 2000 Mmh/Ogg1 gene inactivation results in accumulation of 8-hydroxyguanine in mice. *PNAS* **97** 4156–4161. (doi:10.1073/pnas.050404497)
- Pfaffl MW 2001 A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research* **29** e45. (doi:10.1093/nar/29.9.e45)

- Porcelli AM, Ghelli A, Ceccarelli C, Lang M, Cenacchi G, Capristo M, Pennisi LF, Morra I, Ciccarelli E, Melcarne A *et al.* 2010 The genetic and metabolic signature of oncogenic transformation implicates HIF1 α destabilisation. *Human Molecular Genetics* **19** 1019–1032. (doi:10.1093/hmg/ddp566)
- Rosenquist TA, Zharkov DO & Grollman AP 1997 Cloning and characterization of a mammalian 8-oxoguanine DNA glycosylase. *PNAS* **94** 7429–7434. (doi:10.1073/pnas.94.14.7429)
- Russo MT, De Luca G, Degan P & Bignami M 2007 Different DNA repair strategies to combat the threat from 8-oxoguanine. *Mutation Research* **614** 69–76. (doi:10.1016/j.mrfmmm.2006.03.007)
- Song Y, Driessens N, Costa M, De Deken X, Detours V, Corvilain B, Maenhaut C, Miot F, Van Sande J, Many M-C *et al.* 2007 Roles of hydrogen peroxide in thyroid physiology and disease. *Journal of Clinical Endocrinology and Metabolism* **92** 3764–3773. (doi:10.1210/jc.2007-0660)
- Song Y, Ruf J, Lothaire P, Dequanter D, Andry G, Willemse E, Dumont JE, Van Sande J & De Deken X 2010 Association of duoxes with thyroid peroxidase and its regulation in thyrocytes. *Journal of Clinical Endocrinology and Metabolism* **95** 375–382. (doi:10.1210/jc.2009-1727)
- Shattuck TM, Westra WH, Ladenson PW & Arnold A 2005 Independent clonal origins of distinct tumor foci in multifocal papillary thyroid carcinoma. *New England Journal of Medicine* **352** 2406–2412. (doi:10.1056/NEJMoa044190)
- Tanaka T, Huang X, Jorgensen E, Gietl D, Traganos F, Darzynkiewicz Z & Albino AA 2007 ATM activation accompanies histone H2AX phosphorylation in A549 cells upon exposure to tobacco smoke. *BMC Cell Biology* **8** 26. (doi:10.1186/1471-2121-8-26)
- Volzke H, Ludemann J, Robinson DM, Spieker KW, Schwahn C, Kramer A, John U & Meng W 2003 The prevalence of undiagnosed thyroid disorders in a previously iodine-deficient area. *Thyroid* **13** 803–810. (doi:10.1089/105072503768499680)
- Vonate M, Croce S, Pecchioni C & Papotti M 2002 E2F-1 transcription factor is overexpressed in oxyphilic thyroid tumours. *Modern Pathology* **15** 1038–1043. (doi:10.1097/01.MP.0000028645.36632.A8)

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