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Differential mutation frequency in mitochondrial DNA from thyroid tumours

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Lack of a chromatin structure and histone protection makes mitochondrial DNA susceptible to oxidative damage. Suboptimal DNA repair leads to a higher frequency of mitochondrial mutations, which are associated with aging, carcinogenesis and environmental insult. The instability of the hypervariable region II of the mitochondrial genome was investigated in radiation-associated thyroid tumours, which were diagnosed in children from Belarus after the accident at the Chernobyl nuclear power plant, and from 40 sporadic thyroid tumours from Munich. Two mutations were identified in two out of 126 tumours from Belarus, and eight mutations were found in six out of 40 tumours from Munich. All mutations were deletions or insertions of C in a poly-cytidine (C_7TC_6) microsatellite. The mutation frequency correlated with the age of the patients at surgery. Mutations with the typical pattern of base substitutions following oxidative DNA damage were not identified.

Introduction

The genetic information of mammalian cells is separated into two interdependent genomes. First, the nucleus of a human cell contains the diploid genome of approximately 6 billion base pairs (bp), a small fraction of which codes for 35–70 000 genes. Secondly, up to 1000 mitochondria each harbouring a few copies of circular DNA of 16 569 bp coding for 37 gene products. Mitochondrial DNA (mtDNA) lacks introns and extensive intergenic sequences, and is devoid of histone protection and chromatin structure, which makes its genetic information more vulnerable to DNA damage than the genetic information of the nucleus. The longest stretch of non-coding mtDNA, and also the most variable portion of the human mitochondrial genome, is the main control region for replication, and is composed of the D-loop and the hypervariable regions I and II (1). Within the hypervariable region II (positions 72–337; Cambridge notation, accession no. NC_001807.2; 2) a region of microsatellite-like sequences can be found (positions 208–315). These short tandem repeats, particularly a C-mono-nucleotide track interrupted by a single thymidine at position 310, has been shown to exhibit length polymorphisms among individuals, as well as variations within an individual, which accompany the process of aging (3,4).

Mutations in the mitochondrial genome can give rise to maternally inherited or spontaneous mitochondrial disorders (5). mtDNA aberrations have also been reported for solid tumours and haematological malignancies (3,6–8). However, it is as yet unclear, how an mtDNA variant genome expands and replaces part of (heteroplasmy) or all (homoplasmy) wild-type mtDNA in the affected cells. Observations from pedigrees of families carrying the mitochondria syndrome MELAS (mitochondria encephalopathy, lactic acidosis and stroke-like episodes) and cells *in vitro* from MELAS sufferers, indicate a tendency for homoplasmy (9,10).

As a result of the proximity of mtDNA to reactive oxygen species (ROS) produced in the respiratory chain, higher levels of oxidative damage have been reported in mtDNA compared with levels in nuclear DNA (11). Despite the evidence for base excision repair, direct damage reversal, mismatch repair and recombinational repair—however, not for nucleotide excision repair (12)—the mutation rate in mtDNA is 10-fold higher than in nuclear DNA (13). The accumulation of damage in mtDNA with time (14,15), supports the free radical theory of aging proposed earlier (16), which suggests a link between aging, mitochondrial deterioration and accumulation of oxidative damage.

Exposure of cells to ionizing radiation results in oxidative damage of DNA bases, but also DNA–protein cross-links and single- and double-strand breaks. The response to the DNA damage and the efficacy of DNA repair will determine the fate of the irradiated cell. However, the progeny of survivors of irradiated cells exhibit long-lasting consequences like delayed mutation, clonal heterogeneity, delayed cell death and persisting genomic instability (17). These biological endpoints have been correlated with the perseverance of oxidative stress (18). The resulting mutations in nuclear DNA could predispose the cell to tumour transformation and also lead to a characteristic spectrum of mutations in mtDNA.

Following the interaction of DNA and ROS, generated from endogenous sources or after exposure with ionizing radiation, the most abundantly damaged base is 8-oxodG (19). Although, with a frequency of $<5\%$, this lesion can give rise to G:C $\rightarrow$ T:A transversions. ROS also challenge the dNTP pool causing 8-oxodGTP, which after incorporation opposite dC or dA by DNA polymerases can generate A:T $\rightarrow$ C:G transversions during the next round of replication (20).

Following the accident at the nuclear power plant in Chernobyl in 1986, the population living in the region was exposed to radioactive isotopes from the fall out. A few years after the accident, a dramatic increase in thyroid tumours in children was diagnosed (21) in the areas most contaminated by radioactive iodine (22). These tumours were characterized by chromosomal instability and *RET* proto-oncogene rearrangements, which were thought to be the result of double-strand breaks induced by ionizing radiation (23–27). We have shown recently, that microsatellite instability (MSI) was virtually absent in the majority of radiation-associated thyroid tumours from Belarus, with the exception of aggressive, fast growing thyroid tumours emerging <9 years after the accident.

Abbreviations: mtDNA, mitochondrial DNA; MSI, microsatellite instability; ROS, reactive oxygen species.

However, MSI was a feature of spontaneous thyroid tumours from Munich, which had no radiation history (28,29).

If the exposure of thyroid cells to ionizing radiation causes sufficient genetic disruption to increase the risk of tumourigenesis, then radiation damage to mtDNA could also increase the possibility of mitochondrial mutations. After investigating the stability of mtDNA in 126 thyroid tumours from Belarus, however, no indication of an increased mutation frequency in mtDNA was detected. This was in contrast to a high frequency of mtDNA alterations in 40 spontaneous thyroid tumours from Munich. The analysis of all tumours irrespective of their provenance revealed that the age of the patients at the time of surgery correlated with the mutation frequency of mtDNA.

Materials and methods

Patients, thyroid tumour classification and DNA extraction

Paired samples from tumourous and non-tumourous tissue from 126 thyroid-cancer patients from Belarus (mean age at surgery 16.4 ± 8.7 years) were obtained from the Institute for Radiation Medicine and Endocrinology, Minsk, Belarus. The specimens were classified according to the TNM-gradation by the Thyroid Cancer Centre, Minsk, and the Martha-Maria Hospital, Munich. Of 126 thyroid tumours, 106 were papillary carcinomas (84%), two thyroiditis, two follicular adenomas, two follicular carcinomas, two medullar carcinomas, one nodular goitre, one stroma adenomatosa, one solid carcinoma, seven carcinomas without specified histo-architecture and two specimens without classification.

Samples from tumourous and non-tumourous tissues were also obtained from 40 thyroid cancer patients (mean age at surgery 53.9 ± 15.3 years) with no radiation history from the Martha-Maria Hospital, Munich. The specimens comprised of 16 papillary carcinomas (40%), seven follicular carcinomas, five solid adenomas, three medullar carcinomas, two undifferentiated carcinomas, two carcinomas without specified histoarchitecture, one follicular adenoma, one papillary adenoma and three specimens without classification.

Genomic DNA was extracted from 20 mg of each tumourous and non-tumourous tissue using a DNA isolation kit (Qiagen, Hilden, Germany).

Analysis of mtDNA

Tumourous and non-tumourous samples from 126 patients from Belarus and 40 patients from Munich were used to investigate MSI in mtDNA. Instability of mitochondrial microsatellites was determined by PCR amplification of the *mito-s* fragment (accession no. NC_001807.2, positions 73–337; 2), using primer mito-s-fwd (ACA GGC GAA CAT ACT TAC TAA AGT G, positions 181–205) and mito-s-rev (GTT TGG CAG AGA TGT GTT TAA GTG CTG, positions 353–327). Primer mito-s-fwd was 5′-labelled with fluorescein by the manufacturer (Life Technologies, Karlsruhe, Germany).

DNA amplification was performed in a total volume of 10 µl containing 10–20 ng DNA template in 10 mM Tris–HCl (pH 8), 50 mM KCl, 1.5 mM $MgCl_2$, 100 µM each of dNTPs, 0.5 *U Taq*-DNA polymerase (Life Technologies) and 0.3 nmol of each primer. PCR was based on 36 cycles of 94°C for 45 s, 53°C for 1 min 15 s and 71°C for 45 s with an initial denaturation step of 94°C for 4 min and a final extension at 71°C for 7 min. PCR products were mixed 1:1 with loading buffer (de-ionized formamide, 0.2% dextran blue), denatured at 95°C for 3 min and electrophoresed through a 6% denaturing polyacrylamide gel (Sequagel XR, National Diagnostics, Hull, UK) using an ALF automated sequencer (Amersham Pharmacia, Freiburg, Germany).

Mito-s fragment sizes from all 166 patients were determined by Fragment Analysis Software (Amersham Pharmacia). The differences between fragment length from tumourous and non-tumourous tissue were analysed using SigmaPlot 2000 statistics program module. Values close or outside the 99% prediction interval were considered for sequencing.

Sequencing of mito-s fragment

The *mito-s* fragment from DNA samples form tumourous and non-tumourous tissue from 25 thyroid-cancer patients from Belarus, 12 patients from Munich and from 20 healthy blood donors, were sequenced. Sequences of the hypervariable region II from mitochondria were pre-amplified using mito-p-fwd primer (TCA CCC TAT TAA CCA CTC, positions 14–31) and mito-p-rev (TTG ATG AGA TTA GTA GTA TGG, positions 488–468). The resulting fragment was called mito-p. PCR conditions were identical as described above, with the exception of the annealing temperature of 59°C and a total volume of 30 µl. PCR fragments were purified using a PCR purification kit (Qiagen, Hildesheim, Germany) and checked by 2% agarose gel electrophoresis.

Approximately 100 fmol of mito-p fragment were used as template for the cycle-sequencing reaction using the CEQ DTCS kit (Beckman Instruments, Munich, Germany). The sequencing reaction was primed using 3.2 pmol of mito-fwd-seq oligomer (AGC ATT GCA AGA CGC TGG AGC CGG AGC; positions 84–110) following the manufacturer's instructions. DNA amplification was based on 30 cycles of 96°C for 20 s, 59°C for 20 s and 60°C for 4 min preceded by an initial denaturation step at 94°C for 30 s and terminated by a final extension at 71°C for 4 min. PCR products were ethanol precipitated, washed twice with cold 70% ethanol, dried in a speed vac for 40 min and re-dissolved in sample loading solution provided by the manufacturer. Samples were transferred to Beckman 96-well plates and loaded into a CEQ2000 automated sequencer (Beckman). mtDNA sequences were analysed using Genomax Vector NT3 software (InforMax, Oxford, UK), software provided by the HGMP Resource Centre (Cambridge, UK) and by HUSAR (German Cancer Research Centre, Heidelberg, Germany).

Table I. Mutations in the *mito-s* microsatellite marker in thyroid tumours

Patients from	Tissue	*mito-s* microsatellite CCCCCCCTCCCCCC	Alterations	MSI^{ref}
Belarus: S232	Tum	C_8TC_6	(–1)T(0)	0/26
	Norm	C_9TC_6		
S348	Tum	C_8TC_6	(–1)T(0)	0/26
	Norm	C_9TC_6		
Munich: S10	Tum	C_9TC_7	(+1)T(+1)	2/26
	Norm	C_8TC_6		
S33	Tum	C_9TC_6	(+1)T(0)	0/26
	Norm	C_8TC_6		
S34	Tum	C_8TC_6	(+1)T(0)	0/26
	Norm	C_7TC_6		
S64	Tum	C_9TC_6	(+1)T(0)	1/26
	Norm	C_8TC_6		
S105	Tum	C_7TC_6	(–1)T(0)	0/26
	Norm	C_8TC_6		
S137	Tum	C_9TC_7	(+1)T(+1)	4/26
	Norm	C_8TC_6		

Differences in *mito-s* fragment length were caused by mutations of the microsatellite CCC CCC CTC CCC CC (abbreviated as: C_7TC_6) in the hypervariable region II of human mitochondria from thyroid tumour tissue (Tum) compared with normal tissue (Norm). Deletions or insertions of cytosine to the C_7TC_6 microsatellite sequence were indicated as (–1) or (+1). Microsatellite instability (MSI^{ref}) described the number of altered nuclear microsatellite markers to the number of microsatellites tested, published earlier (29).

Results

The length of the mitochondrial *mito-s* DNA fragment of the hypervariable region II was determined in tumourous and non-tumourous tissue from 126 thyroid-cancers patients from Belarus and 40 patients from Munich, using an ALF automatic sequencer. After seven independent measurements of all 166 patients, differences of *mito-s* fragment sizes between tumourous and non-tumourous tissues of 1 or 2 bp were found in eight patients, which were sequenced.

Comparisons of the *mito-s* sequences from tumourous and non-tumourous DNA samples from these eight patients, revealed that mutations were exclusively found in a microsatellite based on a poly-cytidine sequence (positions 303–315 of the Cambridge notation), interrupted by a single T.

In contrast to the published Cambridge Reference sequence, the 3′-C-repeat of the poly-cytidine microsatellite of all our samples contained six cytosines and not five as the database suggests (hence C_7TC_6). This discrepancy was considered a mistake in the database. All mutations found in the C_7TC_6 microsatellite are summarized in Table I. The thyroid tumours from two patients from Belarus (S323 and S348), each showed deletion of one cytosine of the 5′-C-repeat, whereas most thyroid tumours from patients from Munich showed insertion

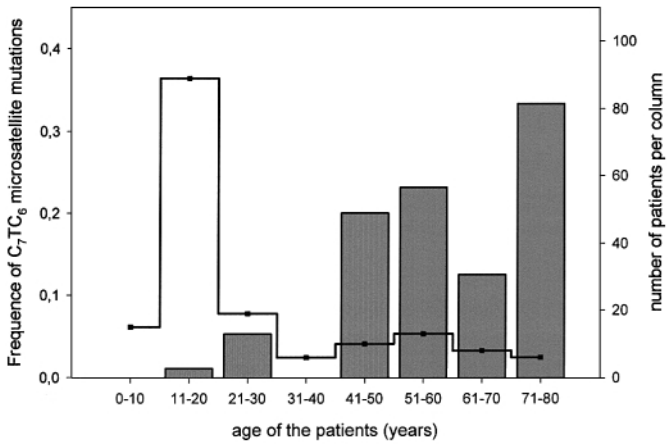

Fig. 1. Mutation frequency of the C_7TC_6 mitochondrial microsatellite and the age of thyroid cancer patients at surgery. Thyroid cancer patients were pooled according to their age at surgery into 10 years intervals and the average frequency of C_7TC_6 microsatellite mutations was calculated (shaded columns, scale on the left ordinate). The step-line gives the number of the patients per interval (scale on the right ordinate).

of one cytosine (with the exception of S105). Only in two tumours from Munich (S10 and S137) the insertion of one cytosine into the 3′-C-repeat was found, remarkably in tumours that had shown increased MSI in an earlier investigation (two, respectively, four altered nuclear microsatellites of 26 markers tested; 29).

Applying the principle of parsimony to these microsatellite mutations, the two sequence alterations found in 126 patients from Belarus represent two mutational events, and the eight sequence alterations found in six out of 40 patients from Munich, represent eight mutational events. The differences in the number of mutational events between the two groups of patients were highly significant ($P = 0.0002$).

The mean age of the patients from Belarus was 16.4 ± 8.7 years and the mean age of the patients from Munich was 53.9 ± 15.3 years. Hence, the difference in the frequency of mutational events in the *mito-s* sequence between the two patient collectives could be the result of the age of the patients. Therefore, the patients were pooled according to their age at surgery into intervals of 10 years and the average frequency of C_7TC_6 microsatellite mutations in each interval was calculated. The result was a seemingly steady increase in the frequency of C_7TC_6 microsatellite mutations in thyroid tumours with the age of the patients (Figure 1, filled columns, scaling left ordinate). The number of patients per interval (step line, scaling right ordinate) varied strongly, because of the high number of thyroid tumours from children from Belarus.

In addition to the sequences obtained from eight patients with C_7TC_6 microsatellite mutations, *mito-s* sequences were also generated from tumourous and non-tumourous tissue from 29 different patients (23 patients from Belarus and six patients from Munich) and from blood samples from 20 healthy controls.

All sequences were aligned and the consensus sequence became the basis of further analysis. Two differences between the *mito-s* consensus and the Cambridge Reference sequences became immediately apparent: at position 83 bp of the *mito-s* consensus sequence, the Cambridge Reference sequence defined adenine, whereas in all samples (including control DNA from healthy donors) guanine was identified. A second difference confirmed results described above: an insertion of cytosine into the C_7TC_5 microsatellite (Cambridge sequence) in position 138 bp of the *mito-s* consensus sequence, was present in all samples to yield the C_7TC_6 microsatellite.

Polymorphisms in the *mito-s* sequences of non-tumourigenic tissue (and healthy controls) were grouped into haplotypes and listed in Table II. The consensus sequence and positions of polymorphisms are given in the top of Table II. Positions of insertions of bases into the Cambridge Reference sequence are indicated by 'x'. The position of the C_7TC_6 microsatellite (position 123–137) is indicated.

The analysis of *mito-s* sequences from 37 patients, revealed that, first, no sequence alterations could be found in tumourous compared with non-tumourous tissue beside the C_7TC_6 microsatellite mutations (data not shown). Secondly, that the C_7TC_6 microsatellite was also a hotspot for polymorphisms (Tables II and III). The ratio of 14 C_7TC_6 microsatellite polymorphisms to 16 polymorphisms in the rest of the *mito-s* fragment was 0.88 for the patients from Belarus and 1.60 for the patients from Munich (8 versus 5, respectively; 0.92 for the healthy controls; see Table III). This excess of microsatellite polymorphisms, as well as microsatellite mutations in patients from Munich, could indicate an increased replication error, or an increased number of replications in the stem cell compartment of these patients before the onset of tumourigenesis, compared with patients from Belarus.

Discussion

The hypervariable region II of the human mitochondrial genome (positions 73–337 of the Cambridge Reference sequence; 2) was found to contain a series of short tandem repeats, which fulfilled the requirements of microsatellite elements. The *mito-s* fragment (positions 181–353) encompassed these microsatellite sequences: $(TTAA)_3$, $(TAA)_3$, $(CA)_3$, A_6 and C_7TC_5 (positions 208–315). Mutations of microsatellite sequences were reported to be restricted to gains and losses of single repeat units (30). Hence, the determination of the *mito-s* fragment length in tumourous and non-tumourous tissue was considered sufficient to establish microsatellite mutations.

Differences in the *mito-s* fragment size of 1 bp between tumourous and non-tumourous samples were found in two out of 126 (or 1.6%) patients from Belarus, and in four out of 40 thyroid tumours from Munich, where in another two tumours 2 bp differences were identified (total of 15%, see Table I). All these mutations were limited to the C_7TC_6 microsatellite. Sequence data from these eight and from a further group of 29 thyroid-cancer patients, showed that none of the tumours harboured base substitutions or combinations of deletions and insertions elsewhere in the *mito-s* fragment. This eliminated the possibility that mutations, which would not have caused size differences, would have gone undetected, and thus would have entailed an underestimation of mutational events.

In an earlier investigation, we found that a nearly identical set of thyroid tumours from Belarus was characterized by stability of nuclear microsatellites (with the exception of a small group of early and aggressive tumours) and that many thyroid tumours from Munich showed MSI (two or more alterations of 26 microsatellites tested). Notably, the two tumours from Munich with two mutations in the mitochondrial C_7TC_6 microsatellite also showed instability of nuclear microsatellites. However, in another eight thyroid tumours from

Table II. Matrix of haplotypes of the *mito-s* sequence from thyroid tumour patients from Belarus and Munich and control individuals

Consensus sequence bp	0	0	0	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1
	0	1	2	4	4	4	5	1	1	2	3	3	3	3	4	4	6	6
	5	5	7	5	7	8	9	3	5	3–9	0	1	2–7	9	0	3	7	8
	G	T	G	G	A	G	T	T	C	C_7	x	x	TC_6	x	G	T	x	C
Haplotypes																		
Belarus: 48	.	.	.	.	.	.	.	.	.	.	–	–	.	–	.	.	–	.
75	.	.	.	.	.	.	.	.	.	.	C	–	.	–	.	.	–	.
80	.	C	.	.	.	.	.	.	.	.	–	–	.	–	.	.	–	.
99	.	.	.	.	.	.	.	.	.	.	C	C	.	–	.	.	T	.
159	.	.	.	.	.	.	C	.	.	.	–	–	.	–	.	.	–	.
214	.	.	.	.	.	.	.	.	.	.	C	C	.	–	.	.	–	.
215	A	.	.	.	.	.	.	.	T	.	C	–	.	–	.	.	–	.
294	.	C	.	A	G	.	.	.	.	.	–	–	.	–	.	.	–	.
323	.	.	.	.	.	.	C	.	.	.	C	–	.	–	.	.	–	.
395	.	C	.	.	.	.	.	.	T	.	–	T	.	–	.	C	–	.
Munich: 11	.	.	.	.	.	.	.	.	.	.	–	–	.	–	.	.	–	.
120	.	.	.	.	.	.	.	.	.	.	C	–	.	–	.	.	T	.
162	.	.	.	.	.	.	.	.	.	.	C	C	.	C	.	.	–	.
10	.	.	.	.	.	.	.	.	.	.	C	–	.	–	.	.	T	del
33	.	C	.	.	.	.	.	.	.	.	C	–	.	–	.	.	–	.
64	.	.	.	.	.	.	C	.	.	.	C	–	.	–	.	.	–	.
105	.	.	.	.	.	.	.	.	.	.	C	–	.	–	.	.	–	.
Controls: A12	.	.	.	.	.	.	.	.	.	.	.	–	.	–	.	.	–	.
A2	A	.	.	.	.	A	.	.	T	.	–	–	.	–	.	.	–	.
A1	.	.	.	.	.	.	.	.	.	.	C	–	.	–	.	.	–	.
B6	.	.	.	.	.	.	.	C	.	.	–	–	.	–	.	.	–	.
B7	.	.	.	.	.	.	.	.	.	.	C	–	.	G	C	.	T	–
C2	.	.	T	.	.	.	.	.	.	.	–	–	.	–	.	.	–	.
C6	.	C	.	.	.	.	.	.	.	.	–	–	.	–	.	.	–	.

The matrix was based on the *mito-s* consensus sequence of all patients and control DNAs. Positions with insertions into the individual's *mito-s* sequence compared with the Cambridge Reference sequence (2) were marked (x), unused positions (–), deletions (del) and no difference (.).

Table III. Disparity of the number of polymorphisms in the C_7TC_6 microsatellite sequence and the rest of the *mito-s* fragment found in normal tissues from patients suffering from thyroid tumours from Belarus (Bel) and Munich (Muc), and healthy controls (Contr)

	Number of individuals	A = Number of polymorphisms in C_7TC_6	B = Number of polymorphisms in *mito-s* (excluding C_7TC_6)	Ratio A/B
Bel	25	14	16	0.88
Muc	12	8	5	1.60
Contr	20	11	12	0.92

Munich with MSI we found no difference in *mito-s* fragment size.

The occurrence of two mutations in the mitochondrial C_7TC_6 microsatellite in 126 thyroid tumours from Belarus was significantly different to the eight mutations found in the 40 thyroid tumours from Munich ($P = 0.0002$). This could be the result of the mean age of the two collectives of patients at surgery. A correlation of the average C_7TC_6 mutation frequency in thyroid tumours with the age of the patients at surgery was identified (Figure 1). The slope of the linear regression line gave 0.22 mutations in the *mito-s* fragment per 50 years or a mutation frequency of 2.5×10^{-5}/bp/year. This mutation frequency was similar to previously published data, which were based on the inclusion of the C_7TC_6 microsatellite (31). Exclusion of the C_7TC_6 microsatellite reduced the mutation frequency of the D-loop ~1000-fold (32,33).

In addition to the mutations found in the C_7TC_6 microsatellite, the sequencing of the *mito-s* fragment of 37 thyroid tumour patients revealed a number of polymorphisms. The average frequency of polymorphisms for the patients from Belarus was slightly elevated compared with patients from Munich. However, the ratio of mutations within the C_7TC_6 microsatellite and the polymorphisms in the rest of the *mito-s* fragment was 0.88 for patients from Belarus and 1.60 for the patients from Munich (healthy controls 0.92; see Table III). This over-representation of microsatellite mutations in thyroid tumours from Munich could be a result of a higher number of cell divisions and the accumulation of replication errors before the onset of tumourigenesis. Hence, the increased number of altered microsatellites found in sporadic thyroid tumours from Munich would reflect the accumulation of mutations in an aged thyroid stem cell and would be proof for the clonality of the tumour rather than a diagnostic marker. The scarcity of C_7TC_6 microsatellite mutations found in thyroid tumours from Belarus would then be a strong indication that a mutator phenotype with MSI would not necessarily be a prerequisite for the process of tumourigenesis.

There remained the possibility that (some of) the polymorphisms could be the result of somatic mutations in thyroid stem cells during early development (see Table II). These mutations could have been the result of oxidative damage by ROS following the exposure to radioactive iodine or as a result of cellular respiration (16,34). The spectrum of base substitutions generated by 8-oxo-guanine would predominantly cause G:C → T:A transversions, whereas ROS attack on the nucleotide pool would yield 8-oxo-GTP, which could result in A:T → C:G transversions (20,35–37). However, none of the polymorphisms found in thyroid-cancer patients showed evidence of such mutations.

The absence of the spectrum of mutations potentially generated by ROS activity is in contrast with the hypothesis of persisting oxidative stress following exposure to ionizing radiation (18). The results presented here indicate that radiation-induced and persisting oxidative stress is limited to nuclear DNA and either the higher levels of oxidative stress protects mitochondria from radiation-induced oxidative stress, or some selection against mitochondria with damaged genomes prevent the fixation of mutations in mtDNA. An indication for such mechanisms can be taken from reports of higher proportion of cells with elevated levels of oxidative stress entering apoptosis or necrosis compared with cells with normal levels (38). Thus, persisting oxidative stress following the exposure to radioactive iodine in the thyroid tumour stem cell compartment could lead to reduced fitness of mitochondrial genomes and their elimination from the population.

Our earlier publication which reported an increased instability of nuclear microsatellites in spontaneous thyroid tumours from elderly patients from Munich, compared with the stability of microsatellites from radiation-induced thyroid tumours from children and young adults from Belarus, were in accordance with the frequency of mutations in mitochondrial microsatellites correlating with the age of the patients at surgery presented here (29). As most tumours are considered to be clonal expansions of a single cell, microsatellite variations reflect the genetic status at the start of the expansion process. However, the genetic distance between the tumourous and the non-tumourous tissue is of no diagnostic use, if genetic alterations have accumulated during the life span of stem cells prior to the onset of tumourigenesis. Genetic alterations could only serve as markers for tumourigenesis if it can be shown that they have been shaped by the process of mutation, selection and apoptosis of a progeny of a tumour stem cell. Here it could be shown, that alterations of mitochondrial microsatellites were age-related genetic events, and would not qualify as markers for tumourigenesis.

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References

1. Hasegawa,M. and Horai,S. (1991) Time of the deepest root for polymorphism in human mitochondrial DNA. *J. Mol. Evol.*, **32**, 37–42.
2. Anderson,S., Bankier,A.T., Barrell,B.G. *et al.* (1981) Sequence and organization of the human mitochondrial genome. *Nature*, **290**, 457–465.
3. Habano,W., Nakamura,S. and Sugai,T. (1998) Microsatellite instability in the mitochondrial DNA of colorectal carcinomas: evidence for mismatch repair systems in mitochondrial genome. *Oncogene*, **17**, 1931–1937.
4. Michikawa,Y., Mazzucchelli,F., Bresolin,N., Scarlato,G. and Attardi,G. (1999) Aging-dependent large accumulation of point mutations in the human mtDNA control region for replication. *Science*, **286**, 774–779.
5. Rothman,S.M. (1998) Mutations of the mitochondrial genome: clinical overview and possible pathophysiology of cell damage. *Biochem. Soc. Sympos.*, **66**, 111–122.
6. Fliss,M.S., Usadel,H., Caballero,O.L., Wu,L., Buta,M.R., Eleff,S.M., Jen,J. and Sidransky,D. (2000) Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. *Science*, **287**, 2017–2019.
7. Penta,J.S., Johnson,F.M., Wachsman,J.T. and Copeland,W.C. (2001) Mitochondrial DNA in human malignancy. *Mutat. Res.*, **488**, 119–133.
8. Bianchi,N.O., Bianchi,M.S. and Richard,S.M. (2001) Mitochondrial genome instability in human cancers. *Muta. Res.*, **488**, 9–23.
9. Kobayashi,Y., Ichihashi,K., Ohta,K., Nihei,K., Kagawa,Y., Yanagisawa,M. and Momoi,M.Y. (1992) The mutant mitochondrial genes in mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) were selectively amplified through generations. *J. Inherit. Metabol. Dis.*, **15**, 803–808.
10. Yoneda,M., Chomyn,A., Martinuzzi,A., Hurko,O. and Attardi,G. (1992) Marked replicative advantage of human mtDNA carrying a point mutation that causes the MELAS encephalomyopathy. *Proc. Natl Acad. Sci. USA*, **89**, 11164–11168.
11. Richter,C., Park,J and Ames,B.N. (1988) Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proc. Natl Acad. Sci. USA*, **85**, 6465–6467.
12. Croteau,D.L., Stierum,R.H. and Bohr,V.A. (1999) Mitochondrial DNA repair pathways. *Mutat. Res.*, **434**, 137–148.
13. Poulton,J., Macaulay,V. and Marchington,D.R. (1998) Mitochondrial genetics '98. Is the bottleneck cracked? *Am. J. Hum. Genet.*, **62**, 752–757.
14. Zhang,C., Liu,V.W.S., Addessi,C., Sheffield,D.A., Linnane,A.W. and Nagley,P. (1998) Differential occurrence of mutations in mitochondrial DNA of human skeletal muscle during aging. *Hum. Mutat.*, **11**, 360–371.
15. Remmen von,H. and Richardson,A. (2001) Oxidative damage to mitochondria and aging. *Exp. Gerontol.*, **36**, 957–968.
16. Harman,D. (1956) Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.*, **11**, 298–300.
17. Morgan,W.F., Day,J.P., Kaplan,M.I., McGhee,E.M. and Limoli,C.L. (1996) Genomic instability induced by ionizing radiation. *Radiat. Res.*, **146**, 247–258.
18. Clutton,S.M., Townsend,K.M.S., Walker,C., Ansell,J.D. and Wright,E.G. (1996) Radiation-induced genomic instability and persisting oxidative stress in primary bone marrow cultures. *Carcinogenesis*, **17**, 1633–1639.
19. Pouget,J.P., Ravanat,J.L., Douki,T., Richard,M.J. and Cadet,J. (1999) Measurement of DNA base damage in cells exposed to low doses of γ-radiation: comparison between the HPLC-EC and comet assays. *Int. J. Radiat. Biol.*, **75**, 51–58.
20. Grollman,A.P. and Moriya,M. (1993) Mutagenesis by 8-oxoguanine: an enemy within. *Trends Genet.*, **9**, 246–249.
21. Demidchik,E., Mrochek,A., Demidchik,Y., Vorontsova,T., Cherstvoy,E., Kenigsberg,J., Rebeko,V. and Sugenoya,A. (1999) Thyroid cancer promoted by radiation in young people of Belarus (clinical and epidemiological features). In Thomas,G., Karaoglou,A. and Williams,E.D. (eds) *Radiation and Thyroid Cancer.* World Scientific, London, pp. 89–96.
22. Jacob,P., Kenigsberg,Y., Zvonova,I. *et al.* (1999) Childhood exposure due to the Chernobyl accident and thyroid cancer risk in contaminated areas of Belarus and Russia. *Br. J. Cancer*, **80**, 1461–1469.
23. Ito,T., Seyama,T., Iwamoto,K.S. *et al.* (1994) Activated RET oncogene in thyroid cancers of children from areas contaminated by Chernobyl accident. *Lancet*, **344**, 259.
24. Fugazolla,L., Pilotti,S., Pinchera,A. *et al.* (1995) Oncogene rearrangements in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear accident. *Cancer Res.*, **55**, 5617–5620.
25. Nikiforov,Y.E., Rowland,J.M., Bove,K.E., Monforte-Munoz,H. and Fagin,J.A. (1997) Distinct pattern of ret oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carinomas in children. *Cancer Res.*, **57**, 1690–1694.
26. Klugbauer,S., Lengfelder,E., Demidchik,E.P. and Rabes,H.M. (1995) High prevalence of RET rearrangement in thyroid tumours of children from Belarus after the Chernobyl reactor accident. *Oncogene*, **11**, 2459–2467.
27. Zitzelsberger,H., Lehmann,L., Hieber,L. *et al*. (1999) Cytogenetic changes in radiation-induced tumours of the thyroid. *Cancer Res.*, **59**, 135–140.
28. Richter,H.E., Lohrer,H.D., Hieber,L., Kellerer,A.M., Lengfelder,E. and Bauchinger,M. (1999) Microsatellite instability and loss of heterozygosity in radiation-associated thyroid carcinomas of Belarussian children and adults. *Carcinogenesis*, **20**, 2247–2251.
29. Lohrer,H.D., Braselmann,H., Richter,H.E., Jackl,G., Herbeck,J., Hieber,L., Kellerer,A.M. and Bauchinger,M. (2001) Instability of microsatellites in radiation-associated thyroid tumours with short latency periods. *Int. J. Radiat. Biol.*, **77**, 891–899.
30. Weber,J.L. and Wong,C. (1993) Mutation of human short tandem repeats. *Hum. Mol. Genet.*, **2**, 1123–1128.
31. Sigurdardottier,S., Helgson,A., Gulcher,J.R., Stefanson,K. and Donnelly,P. (2000) The mutation rate in human mtDNA control region. *Am. J. Hum. Genet.*, **66**, 1599–1609.

32. Horai,S., Murayama,K., Hayasaka,K., Matsubayashi,S., Hattori,Y., Fucharoen,G., Harihara,S., Park,K.S., Omoto,K. and Pan,I.H. (1996) mtDNA polymorphisms in East Asian populations, with special reference to peopling of Japan. *Am. J. Hum. Genet.*, **59**, 579–590.
33. Fucharoen,G., Fucharoen,S. and Horai,S. (2001) Mitochondrial DNA polymorphisms in Thailand. *J. Hum. Genet.*, **46**, 115–125.
34. Cortopassi,G.A. and Arnheim,N. (1990) Detection of a specific mitochondrial DNA deletion in tissues of older humans. *Nucleic Acids Res.*, **18**, 6927–6933.
35. Tajiri,T., Maki,H. and Sekiguchi,M. (1995) Functional cooperation of MutT, MutM and MutY proteins in preventing mutations caused by spontaneous oxidation of guanine nucleotide in *Escherichia coli*. *Mutat. Res.*, **336**, 257–267.
36. Le Page,F., Guy,A., Cadet,J., Sarasin,A. and Gentil,A. (1998) Repair and mutagenic potency of 8-oxoG:A and 8-oxoG:C base pairs in mammalian cells. *Nucleic Acids Res.*, **26**, 1276–1281.
37. Gasparutto,D., Bourdat,A.-G., D'Ham,C., Duarte,V., Romieu,A. and Cadet,J. (2000) Repair and replication of oxidized DNA bases using modified oligodeoxyribonucleotides. *Biochimie*, **82**, 19–24.
38. Limoli,C.L., Hartmann,A., Shephard,L., Yang,C.-R., Boothman,D.A., Bartholomew,J. and Morgan,W.F. (1998) Apoptosis, reproductive failure and oxidative stress in Chinese hamster ovary cells with compromised genomic integrity. *Cancer Res.*, **58**, 3712–3718.

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