Large Deletions in Mitochondrial DNA in Radiation-associated Human Thyroid Tumors


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ABSTRACT

Paired DNA samples of tumor and normal thyroid tissue from adult patients possibly exposed to radioactive Chernobyl fallout ([11 cases of papillary thyroid carcinoma (PTC) and 6 follicular adenomas] and from control samples ([9 PTC occurring in Japanese patients] were examined for the relative mitochondrial DNA (mtDNA) content, prevalence and level of common deletion (CD), and large-scale deletions in mtDNA. Elevated relative mtDNA content as estimated by real-time PCR was found in tumor tissue in most cases, but no significant correlation with the level of radioiodine contamination of patients’ residency nor with clinicopathological data were found. CD was detected in every DNA specimen from all types of tissue regardless of the presence of oxyphilic cell changes. Elevated level of the CD was predominantly found in tumor tissue of the radiation-associated group but not in sporadic PTC. No correlation was noted with clinicopathological parameters, radioiodine contamination, and relative mtDNA content. The quantity of large-scale deletions in mtDNA was elevated in most tumor tissues, especially in the radiation-associated group and tended to correlate with the level of radioiodine in PTC. In contrast to sporadic PTC, highly significant positive correlation between the presence of large scale mtDNA deletions and relative mtDNA content was found in radiation-associated tumors (P = 0.001 and P = 0.019 in PTC and follicular adenoma, respectively). Normal tissue displayed the inverse tendency. No association with level of the CD was found in either group of cases. Concordant increase of both relative mtDNA content and number of mtDNA deletions was detected more often in radiation-associated PTC than in sporadic PTC. Thus, simultaneous determination of the number of large-scale mtDNA deletions and relative mtDNA content may be useful to elucidate molecular distinct features of radiation-associated thyroid tumors.

INTRODUCTION

mtDNA3 is recognized to evolve 10–100 times faster than nuclear DNA because of a number of reasons. Those most often reported are the aggressive environment reach in reactive oxygen species specific to mitochondrion, increased infidelity of mtDNA polymerase γ, slippage of mitochondrial systems of DNA repair, particulars of mtDNA structure, spatial proximity to mitochondrial membrane, and peculiarities of mtDNA replication and transcription (1–8).

Ionizing radiation is a potent genotoxic factor that may contribute to the excessive mutagenesis of nucleic acids, and both point mutations and large-scale deletions in mtDNA have been shown to arise after exposure (9–15). The study of insertions at this mononucleotide repeat (20).

The results of systematic analysis of mtDNA in various thyroid diseases demonstrated a significant difference in the incidence of point mutations between cases with thyroid pathology and control tissues, especially between thyroid carcinoma cases and control population (26). Furthermore, statistical significance was found in the differential distributions of all sequence variant types between carcinomas and controls in contrast to benign tumors and nonneoplastic diseases. Direct sequencing of 70% of the mitochondrial genome undertaken to evaluate relative efficacy of systems of mtDNA repair demonstrated higher rate of radiation-induced strand breaks and longer persistence of DNA alterations in mitochondria than in the nucleus (16). Consistently, mtDNA was found to be a critical cellular target for reactive oxygen species such as hydrogen peroxide (17). Complex examination of the spectrum of deletions in mtDNA by long-range PCR has provided evidence of the usefulness of this index as of a biomarker of UV exposure in skin (15).

Mitochondrial genetic instability has been demonstrated to occur in a large variety of human malignancies at nearly any level of complexity (Ref. 18 for review). Homoplasmic or heteroplasmic mtDNA point mutations were found with a prevalence ranging from about two-thirds of cases in colorectal carcinoma to virtual absence of those in testicular tumors. Heteroplasmic CD was found in breast carcinoma, preferentially in postmenopausal patients, perhaps attributable to aging cells (19). Recently, a mutational hot spot in primary tumors has been specifically associated with highly polymorphic homopolymeric C stretch within the D-loop (bases 311–315) of mtDNA. Over one-fifth of the tumors showed somatic mutations, deletions, and insertions at this mononucleotide repeat (20).

In thyroid diseases, alterations in mtDNA studied in Hashimoto’s thyroiditis were defined as a 4977-bp long CD associated with or independent from concomitant defects of cytochrome-c oxidase in oxyphilic cells in affected but not in normal regions of the gland (21). A parallel study has demonstrated that this mutation may be found not only in Hashimoto’s thyroiditis but also in Hurthle cell carcinoma, as well as in multinodular goiter and FA with or without oxyphilic cell changes (22). In that series of cases, normal thyroid tissue, with isolated instance, was devoid of such kind of mutation regardless of the presence of mild lymphocytic thyroiditis, and in the most frequently occurring thyroid malignancy, PTC, the CD was not found. At somewhat variance to these findings, a previous study of a series of 12 oncocytic thyroid tumors did not reveal increased frequency of mtDNA deletions in tumors compared with the controls (23). Another earlier study showed no indication of heteroplasmy of mtDNA or other gross alterations as assessed by restriction analysis (24). In a recent report by Maximo et al. (25), the presence of CD was found in varying percentage in any histopathological variant of thyroid tumors with the exclusion of follicular thyroid carcinoma and anaplastic cancers. The latter variant was not analyzed in the cited work. Besides follicular carcinoma and FA, in 12.5–33.3% of studied cases, normal thyroid tissue adjacent to the tumor has been found to have a small proportion of CD in all other thyroid tumor variants.

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3 The abbreviations used are: mtDNA, mitochondrial DNA; PTC, papillary thyroid carcinoma; FA, follicular adenoma of thyroid; CD, common deletion in mitochondrial DNA.

ABSTRACT

Paired DNA samples of tumor and normal thyroid tissue from adult patients possibly exposed to radioactive Chernobyl fallout ([11 cases of papillary thyroid carcinoma (PTC) and 6 follicular adenomas] and from control samples ([9 PTC occurring in Japanese patients] were examined for the relative mitochondrial DNA (mtDNA) content, prevalence and level of common deletion (CD), and large-scale deletions in mtDNA. Elevated relative mtDNA content as estimated by real-time PCR was found in tumor tissue in most cases, but no significant correlation with the level of radioiodine contamination of patients’ residency nor with clinicopathological data were found. CD was detected in every DNA specimen from all types of tissue regardless of the presence of oxyphilic cell changes. Elevated level of the CD was predominantly found in tumor tissue of the radiation-associated group but not in sporadic PTC. No correlation was noted with clinicopathological parameters, radioiodine contamination, and relative mtDNA content. The quantity of large-scale deletions in mtDNA was elevated in most tumor tissues, especially in the radiation-associated group and tended to correlate with the level of radioiodine in PTC. In contrast to sporadic PTC, highly significant positive correlation between the presence of large scale mtDNA deletions and relative mtDNA content was found in radiation-associated tumors (P = 0.001 and P = 0.019 in PTC and follicular adenoma, respectively). Normal tissue displayed the inverse tendency. No association with level of the CD was found in either group of cases. Concordant increase of both relative mtDNA content and number of mtDNA deletions was detected more often in radiation-associated PTC than in sporadic PTC. Thus, simultaneous determination of the number of large-scale mtDNA deletions and relative mtDNA content may be useful to elucidate molecular distinct features of radiation-associated thyroid tumors.

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revealed a significant difference in the prevalence of nonsilent mutations in complex I genes between FA and PTC (25). Importantly, normal parenchyma adjacent to the tumor also frequently harbored mtDNA sequence variants in complex I and IV regions demonstrating that thyroid tumor is not the only type of tissue that may possess mutated mtDNA.

Taking into account data on mtDNA in various thyroid diseases and the known fact that thyroid is a target for radiation (Ref. 27 for review), we attempted to address a question whether possible exposure to radiiodine might lead to the increased mutational rate in mtDNA in thyroid of people affected by the Chernobyl fallout. The massive amount of radioactive isotopes released into the environment after the destruction of a reactor unit in April 1986, of which only $^{131}\text{I}$ accounted for some $1.3\text{--}1.8 \times 10^{18} \text{Bq}$, is the most likely reason for this event.

The level of mtDNA/nuclear DNA ratio determined by the described assays.

**Materials and Methods**

**Patients and Tissues.** Seventeen snap-frozen postoperational specimens of tumor and matched normal thyroid tissues were available for the study. Of these, 11 were PTC and 6 FA as confirmed by two independent pathologists (A. Y. A., E. F. L.). Tissues were collected at Medical Radiological Research Center (Okininsk, Russia) from 36–50-year-old patients (mean age, 41.0 ± 1.2 years, 2 males and 15 females) undergoing surgery for thyroid tumor. Informed consent was obtained from every individual according to ethic guidelines of the Nagasaki University Hospital.

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solution from the Thermo Sequenase Cycle Sequencing kit (Amersham Pharmacia Biotech United Kingdom Limited, Little Chalfont, United Kingdom), denatured for 5 min at 95°C, quickly chilled in ice-water bath, and then 2 μl of the mixture was loaded on regular 6% sequencing gel in 5500 DNA Sequencer (Hitachi Instruments Service Co., Ltd., Japan). Molar weight standard ranging 50–500 bases in 50-base increments was a commercially available Texas Red-labeled mixture of single-stranded DNA oligonucleotides (Amersham Pharmacia Biotech United Kingdom Limited). Gel images were generated with DNAStar software package (Hitachi). The number of bands per lane was calculated by visual confrontation of gel images with absorbance profile obtained for each lane with a NIH Image program (Bethesda, MD).

To determine the primary structure of aberrant products, 10 μl of the PCR reaction were resolved in 6% native polyacrylamide gel and stained with SYBR Green I dye. Arbitrarily picked bands were excised from gel, and DNA elution was performed by heating the sample at 95°C for 10 min in 100 μl of double-distilled water. Extracted material was precipitated with ethanol in the presence of 1 μl of 20 mg/ml glycogen solution (Roche, Mannheim, Germany) and 10 μl of 7.5 mM ammonium acetate. The pellet was reconstituted in 3 μl of water, and 1 μl of the solution was used to directly clone the PCR product into pGEM–T Easy vector (Promega, Madison, WI). Sequencing was performed with Thermo Sequenase Cycle Sequencing kit (Amersham Pharmacia Biotech United Kingdom Limited).

Southern Blotting. Total DNA (2.5 μg) from matched normal and tumor tissues was digested with PvuII to linearize mtDNA, resolved in 0.7% agarose gel, denatured, transferred onto positively charged nylon membrane (Roche), and hybridized overnight in ULRAGb buffer (Ambion, Austin, TX) at 42°C with two PCR probes covering nearly full-length mtDNA (Table 1). Probes (6.7 and 8.9 kb of mtDNA) were synthesized in PCR reactions with LA Taq (Takara, Otsu, Japan) using 25 ng of total human DNA as a template. Before the addition to hybridization solution, probes were denatured for 1 min at 95°C and quickly chilled in an ice-water bath. Detection was performed with alkaline phosphatase-conjugated F(ab')2 fragments of antibody to digoxigenin and CDP-Star reagent (Roche) according to manufacturer’s recommendations.

Primary Thyroid Cell Culture and in Vitro Irradiation. Primary human thyroid cell cultures were established as described previously (34). Cells were maintained in 2:1 mixture of F12 Nutrient Mixture and DMEM supplemented with 3% fetal bovine serum and 1× penicillin-streptomycin (all reagents from Invitrogen Life Technologies, Inc., Paisley, United Kingdom) in 5% CO2 humidified atmosphere. Irradiation with X-rays was performed in single doses ranging from 0.5 to 5 Gy with EXS-300 irradiator (200 kV, 15 mA, filter: 0.5 mm aluminum, 0.939 Gy/min; Toshiba, Tokyo, Japan). Exposed cells were allowed to recover for 48 h, and total DNA was isolated by standard proteinase K/phenol-chloroform extraction procedure.

Statistical Analysis. To analyze the results, simple regression model of the GLM procedure, double-sided Fisher’s exact test, and nonparametric Mann-Whitney test were used whenever applicable (SAS/STAT software package; SAS Institute, Inc., Cary, NC; Ref. 35). Statistical significance was considered P not exceeding 0.05.

RESULTS

Relative mtDNA Content in Normal and Tumor Thyroid Tissue. Relative mtDNA content evaluated by real-time PCR was found to be elevated in most tumor tissues of the radiation-associated group. As shown in Fig. 1A, 14 of 17 radiation associated cases (9 of 11 in PTC and 5 of 6 in FA) displayed higher mitochondrial to nuclear DNA ratio representative of frequent increase in the mtDNA levels in thyroid tumors. Statistical analysis did not reveal association of the mtDNA level with pathological characteristics such as size and extent of the tumor in this set of cases. Relative mtDNA content tended to positively correlate with the level of radioiodine contamination only in tumor tissues of PTCs, and with the rejection of one outlier, the association became statistically significant in linear approximation (P = 0.014; Fig. 1B). The mtDNA content in normal thyroid tissue of PTC cases, as well as in both types of tissue of FA cases, did not correlate significantly with radioiodine contamination (Fig. 1, B and C). Furthermore, there was a weak tendency toward the decrease of this index in cases originating from regions with higher level of contamination.

Control group of sporadic PTCs developed in Japanese patients included 5 of 9 cases (55.6%) in which relative mtDNA content was higher in the tumor as compared with normal tissue (Fig. 1A). This proportion does not result in statistical difference between the radiation-associated and sporadic groups in terms of prevalence of cases with elevated mtDNA content in the tumor (P < 0.336 by Fisher's exact test).

CD in mtDNA. The detection of CD between the origins of replication of light and heavy mtDNA strands was performed by PCR amplification with two sets of primers. One primer pair localized inside of the region referred to as CD yields a 142-bp amplicon corresponding to wild-type mtDNA (22). In case the 4977-bp deletion is present, a 262-bp PCR product is generated as determined by another pair of primers annealing to the fragments flanking the deleted region (see Table 1 for details).

Examination of normal and tumor thyroid tissue demonstrated the occurrence of CD in all DNA specimens tested, both in radiation-associated and sporadic groups (Fig. 2). Some of the DNA samples displayed additional PCR products, the size of which was different from the band corresponding to the CD. More often, but not necessarily, these PCR fragments were larger than the 262-bp product indicative of the presence of low abundant truncated mtDNA templates with diversely deleted fragments.

Visual and densitometric examination of ethidium bromide-stained agarose gels and of polyacrylamide gels stained with SYBR Green I

Fig. 1. Proportion of cases with elevated relative mtDNA content in the tumor tissue in radiation-associated (Rad) and sporadic (Spor) thyroid tumors (A) and correlation of relative mtDNA content with level of radioiodine contamination of patient’s residency (B and C) in radiation-associated group. Open symbols and dotted line correspond to normal thyroid tissue; closed symbols and solid line correspond to normal thyroid tissue. Arrow in graph B marks an outlier rejection of which leads to significant elevation of the confidence.
did not reveal association of the intensity of the CD band after 20, 25, and 30 amplification cycles with clinicopathological parameters of tumors in this series of cases. Level of the CD was usually higher in the tumor tissue compared with normal, although not in all cases in a radiation-associated group, 8 of 11 (72.7%) in PTC and 4 of 6 (66.7%) in FA, whereas in a group of sporadic PTC, there were only 3 of 9 (33.3%) of such cases as estimated by real-time PCR (Fig. 3A). Prevalence of cases with higher level of CD content in tumor tissue in spite of being diverse between radiation-associated and sporadic PTCs and, more generally, between radiation-associated and sporadic cases was not significantly different between both pairs of groups \((P = 0.175\) and \(P = 0.095\), respectively, by Fisher’s exact test). SYBR Green real-time PCR analysis of the level of CD with respect to radioiodine contamination and mtDNA content did not demonstrate significant association between these indexes (Fig. 3B and C). Weak tendency to the positive correlation between mtDNA content and the CD levels was observed only in tumor tissue of radiation-associated PTC (Fig. 3D–F). Thus, in PTC and FA, the CD is quite prevalent and is unlikely to be representative of thyroid tumor disease.

Large-scale Deletions in mtDNA. Revelation of alternate PCR products coamplified along with CD prompted us to establish a technique that would allow analysis of noncanonic derivatives. To increase the detection limit of the assay, we developed an approach combining high resolution and sensitivity of a sequencing system with multiplex PCR using a set of primers, one of which (forward) was 5’ labeled with fluorescent dye and two reverse primers were located 5.2 and 8.3 kb separate from it. Presumably, short elongation time of PCR reaction would allow only those templates with deleted interstitial region(s) between forward and one of the reverse primers to be amplified. This condition also satisfies the requirement of the size of PCR fragments to be resolved in a sequencing gel not to exceed 1 kb.

Examination of DNA samples extracted from normal and tumor tissue of series of cases demonstrated a large variety of aberrant mtDNA molecules occurring in virtually each specimen (Fig. 4). The average number of PCR products per DNA sample varied from 5 to >60, and samples yielding higher band score reproducibly demonstrated it compared with lower ones, although exact number of bands and spectrum of PCR products varied between the PCR batches. In radiation-associated PTCs, 9 DNA samples of 11 displayed higher average numbers of mtDNA deletions in tumor tissue than in normal (Fig. 5A), and in FA, there was equal number (3 and 3) of samples with elevated or diminished mutation scores in the tumor DNA. Statistical analysis did not reveal association of average number of noncanonic PCR products with clinicopathological parameters.

Correlation between the number of mtDNA deletions and level of \(^{131}\)I contamination was nonsignificant both in radiation-associated PTC and FA (Fig. 5B and C). Only a tendency to the increase in mtDNA deletions with the increase of contamination level was observed in tumor tissues of PTCs. On the contrary, a highly significant correlation with mtDNA content in tumor tissue of radiation-associated cases, especially in PTC, was found (Fig. 5E). Even with the
rejection of an outlier, association was significant ($P = 0.044$). In FA, this correlation existed as well (Fig. 5F), albeit, it was weaker and became less significant if one or two outliers were rejected ($P = 0.052$ and $P = 0.321$, respectively). In normal tissue of FA, the number of mtDNA deletions tended to decrease with increased mtDNA level similar to PTC, thus demonstrating strictly different regularity as compared with the tumor.

In sporadic PTCs, distribution of cases with larger number of mtDNA deletions between tumor and normal tissue was not strongly biased to the tumors as in radiation-associated series. Of nine cases, there were four of each displaying either increased or decreased mtDNA mutational scores in tumor tissue, and one case had equal average number of mtDNA deletions in normal and tumor tissue (Fig. 5A). Although there was no statistically significant difference between the proportion of cases with elevated mtDNA mutation score in the tumor tissue of radiation-associated and sporadic cases ($P < 0.08$), the tendency may imply some positive association between the number of mtDNA deletions and possible radiation exposure.

Correlation of the number of mtDNA deletions with relative mtDNA content in sporadic PTCs was insignificant (Fig. 5D), with the main trend preserved, i.e., increased mutation score in tumors with elevated mtDNA level and vice versa in normal tissue found in the radiation-associated group.

No correlation between the level of CD and number of mtDNA deletions was observed in any group of tissues under investigation (Fig. 5, G–I). This finding is of particular interest as it suggests that the formation of the CD and other mtDNA deletions are not associated and may arise via different mechanisms.

To verify that the bands revealed on sequencing gel and interpreted as large-scale mtDNA deletions were not artifacts, PCR mixtures were resolved in acrylamide gel, and several arbitrary selected visible bands of different intensity were excised and products were cloned and sequenced. Every specimen was found to be authentic mtDNA fragment with large deletions, the size of which clustered ~5 and 8 kb (Table 2). Size of characterized deletions, however, is determined by the technical design of experimental approach and obviously depends on the primer set used. No preferential association of the size of deletions with tumor or normal tissue, as well as with benign or malignant thyroid disease, was found. Analysis of the breakpoint structure demonstrated that in every case there were short, 2–7 bases long patches of microhomology presented by direct or inverted direct repeats, sometimes even more than one, located exactly at or a few bases apart from the breakpoint. Thus, taken together, results testify that the method of revelation of mtDNA deletions used in this work was not severely compromised by technical artifacts.

Given there was an association between mtDNA content and the deletion score, we performed Southern blotting of the genomic DNA to discriminate whether the observed increase of mtDNA level may be because of the increase in the content of mutated mtDNA molecules. Results of this experiment shown in Fig. 6 suggest that truncated mtDNA molecules account for rather a minute proportion of total mtDNA. The signal from the 11.6-kb bands representing mtDNA with CD was very weak compared with wild-type mtDNA, and in agarose and sequencing gels (Figs. 2 and 4), intensity of the CD bands was considerably more profound than that of the vast majority of other products.

**Association of mtDNA Deletions with Radiation Dose.** Accumulation of mtDNA deletions was assessed in primary cultures of human thyrocytes irradiated with 0.5–5 Gy of X-rays 2 days after the expo-
This model was chosen to allow irradiated cells to recover and to largely complete the DNA repair during certain time after irradiation thought to be representative of radiation-induced thyroid tumors developing at least several years after the exposure. As demonstrated in Fig. 7A, there was a marginally significant dose-dependent increase of mtDNA deletions in exposed cells with an increment of 5.8 additional mutant molecules/Gy. Actual shape of the dose-response curve was rather better characterized by a logarithmic growth of the mutation score reflecting technical limitations of the used assay and, possibly, the complexity of biological events underlying the formation of aberrant DNA molecules. Note also that even nonexposed cells displayed a certain number of low abundant large-scale mtDNA deletions perhaps comprising a natural background.

The level of the CD and mtDNA content in same cultures did not undergo significant changes 2 days after irradiation as estimated by real-time PCR (Fig. 7B). Hence, ionizing radiation can generate large-scale DNA deletions without disturbing the mtDNA level in cultured thyrocytes during short time after the exposure. Therefore, at least some of the aberrant mtDNA molecules revealed in the DNA specimens extracted from respective tissues might be attributed to the effect of radiation.

**DISCUSSION**

In this study, we attempted to address several questions of mtDNA status in a series of human thyroid tumors to elucidate whether changes in mtDNA can be linked to possible exposure to radiation fallout after the Chernobyl accident. To emphasize the difference between pediatric and adult cases of post-Chernobyl thyroid tumors, we used the term radiation associated instead of radiation induced as more appropriate in relation to the adult series included in our study.

Evaluation of relative content of mtDNA by real-time PCR allows an accurate estimate of this parameter given there are no gross alterations of mtDNA observed in some mitochondrial diseases (36–39). In thyroid diseases, such alterations have not been reported, therefore, this approach could reliably be applied to our series of specimens. According to the results, the number of mtDNA copies was elevated in the tumor tissue in a significant proportion of cases, irrespective of their malignant or benign nature, being slightly higher in the radiation-associated group. Although experimental design of the study did not allow discrimination between the elevation of mtDNA copies per se and increase in the number of mitochondria, it is more probable that the latter circumstance is responsible for the observed phenomenon. The elevated number of mitochondria has been demonstrated in most of thyroid cancers as assessed by ultramicroscopy (40), and particularly in oncocyctic tumors, it might be considered a hallmark accompanied by structural abnormalities of the organelles (Ref. 41 for review). The mechanism of regulation of a number of mitochondria in human cells is elusive, however, in Hurthle cell tumors, it is likely that the deficiency in oxidative phosphorylation associated with coupling impairment in mitochondria (42, 43) is one of possible
Table 2 Sequence of the breakpoints in deletion-type mtDNA PCR products

<table>
<thead>
<tr>
<th>5' Flanking sequence</th>
<th>Deleted region</th>
<th>3' Flanking sequence</th>
<th>Size of deletion, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>8221</td>
<td>ttatatttcc</td>
<td>aggctagcat</td>
<td>13475</td>
</tr>
<tr>
<td>CCCCATGCCTCTAGA</td>
<td>gttctctatt</td>
<td>tcacccccaa</td>
<td>5234</td>
</tr>
<tr>
<td>8198</td>
<td>agcctagcct</td>
<td>tcccccctcc</td>
<td>5167</td>
</tr>
<tr>
<td>8239</td>
<td>tttgaaatag</td>
<td>aagataacct</td>
<td>13410</td>
</tr>
<tr>
<td>8256</td>
<td>ggcctgatt</td>
<td>attatcggaa</td>
<td>5159</td>
</tr>
<tr>
<td>8233</td>
<td>aaaaaatcct</td>
<td>tctacccaaac</td>
<td>5149</td>
</tr>
<tr>
<td>8271</td>
<td>cccctctacga</td>
<td>atatcggcct</td>
<td>5138</td>
</tr>
<tr>
<td>8239</td>
<td>tttgaaatag</td>
<td>caagcctcttc</td>
<td>5100</td>
</tr>
<tr>
<td>8259</td>
<td>tataccttag</td>
<td>tacatcttat</td>
<td>5099</td>
</tr>
<tr>
<td>8307</td>
<td>aggctagctt</td>
<td>tttaaccctc</td>
<td>5073</td>
</tr>
<tr>
<td>8362</td>
<td>aagataacct</td>
<td>cacatccttc</td>
<td>5030</td>
</tr>
<tr>
<td>8170</td>
<td>ttgctcggaa</td>
<td>tttaaccctc</td>
<td>8162</td>
</tr>
<tr>
<td>8244</td>
<td>aataggcccc</td>
<td>aatgaccccc</td>
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<tr>
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<td>cccctctgag</td>
<td>gttccctctgg</td>
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</tr>
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<td>ttagattgac</td>
<td>aacacccccctcc</td>
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<td>8290</td>
<td>tttactatctt</td>
<td>tctagatgcc</td>
<td>8048</td>
</tr>
<tr>
<td>8257</td>
<td>aggccctagt</td>
<td>acccc gaat</td>
<td>7984</td>
</tr>
<tr>
<td>8307</td>
<td>aagataacct</td>
<td>cacatccttc</td>
<td>7904</td>
</tr>
</tbody>
</table>

Abb Underlined are direct repeats, inverted direct repeats, or short homologous sequences. In cases when direct repeats locate exactly at or immediately flank the breakpoints, position of the junction is tentative.

reasons. Accordingly, increase in the number of mitochondria may occur as a compensatory response to insufficient energy production and is mediated by a concordant expression of nuclear and mitochondrial genes acting in a putative feedback manner (Ref. 24, for review Refs. 44, 45). Concerted expression of genes involved in the mtDNA replication and mitochondrial function may be partly achieved by the recruitment of common transcription factors such as NRF-1, NRF-2, and PGC-1 directing the expression of subunits of the oxidative phosphorylation system as well as of mtTFA and MRP genes (46–48). Other factors like mitochondrial nucleoside pool, particularity of mtDNA primary structure and cell cycle, may also influence the mtDNA copy number (49–52). Renal and thyroid oncotic tumors have been shown to have 5-fold increased level of mtDNA (23), which is in line with a general concept of the association of mtDNA copy number with mitochondrial mass (53). We, therefore, tend to interpret the observed elevation of mtDNA levels in most of tumor tissues as a consequence of the increased organelle number, examination of the cause of which may involve a matter of a separate study.

The increase in mtDNA level apparently was not radiation specific because there was an elevation of mtDNA content in approximately a half of sporadic PTC cases, and no significant correlation with level of radioiodine contamination of the place of patients’ residency was observed. However, level of environmental radioiodine pollutant has only conditional correctness as an unbiased indicator of thyroid exposure, and therefore, this inference may be a subject of revision in the future provided the study is conducted on DNA samples derived from cases with known individual thyroid dose or there is a larger series of cases. Latter approach seems to be a realistic one since the establishment of the Post-Chernobyl Thyroid Tissue Bank (54).

CD in mtDNA is accepted to be inversely dependent on the mitotic activity of the tissue and tends to accumulate with time preferentially in tissues with lower turnover (55, 56). Presence of the CD in thyroid tissues was found in Hashimoto’s thyroiditis, Hurthle cell tumors, multinodular goiter, PTC, and FA, although data from different groups are not always in line with each other (21–23, 25). In the cases included in our study, occurrence of the CD was found in every DNA sample extracted from either normal or tumor tissue irrespectively the sample was from cases with known individual thyroid dose or there is a larger series of cases. Latter approach seems to be a realistic one since the establishment of the Post-Chernobyl Thyroid Tissue Bank (54).
tissue was involved only in 12.5% in PTC and completely devoid of the CD in FA (25). In our cases, high prevalence of the CD was dissociated from oxyphilic cell changes according to histological examination, at least such changes were not seen in sections used for the diagnostics. Early steps of oxyphilic change represented by appreciable level of the CD may not necessarily be accompanied by typical morphological characteristics of oxyphilic cells (21, 22, 57), however, it is quite unlikely that all of the tissues under our investigation involved such cases. We, therefore, assume that some differences in techniques used by different laboratories and intrinsic features of samples available for each study (e.g., fresh frozen tissue versus formalin fixed, age and ethnic background of patients, and so on) may account for variant reading of the problem. Data obtained in our experiments show that prevalence of the CD in thyroid tissue may be very high, up to 100%, yet the abundance of mtDNA molecules with CD is extremely low compared with wild-type mtDNA as seen from results of Southern blotting. This statement is concordant with quantitative estimates of the CD content in PTC and FA tumors found not to exceed 0.5 ± 0.7% of total mtDNA (25).

Statistical analysis demonstrated that level of the CD in our series of cases was not associated with either radioiodine contamination of the territory or mtDNA content in the tissue. Although from model cell cybrid experiments it is known that increased content of the mtDNA with CD and chemically induced oxidative stress may cause a proportional increase of relative mtDNA content and mitochondrial mass (58), it is most likely that in cases under investigation, low relative content of the CD was not sufficient to evoke phenotypic changes. Thus, our data support the notion that accumulation of the CD may precede the increase in mtDNA rather than elevated level of mtDNA leading to the escalation of the CD.

In X-ray-irradiated primary thyrocytes, changes of the CD level were not observed during 2 days after exposure in our experiments. The probable reason for this might be high radioreistance of human thyrocytes demonstrated in our earlier works (59, 60) and relatively low doses of X-ray radiation used in this study in contrast to observation in human squamous cell carcinoma lines and in SV40-transformed human fibroblasts of patient with ataxia telangiectasia (11). Induction of the CD in thyrocytes is unlikely to be a direct immediate consequence of the exposure that may take place shortly after irradiation as a result of double-strand DNA break repair. Its generation and expansion may be driven by other mechanisms that involve the cooperative effect of defective mitochondrial systems of DNA repair, structural features of the deletion prone mtDNA region, and enhanced replicative rate of mtDNA with CD (44, 61) rather than because of exclusive pressure of genotoxic radiation-associated factors. Along with that, latter agents may also have certain impact as demonstrated by preferential accumulation of the CD in open skin areas and in cultured cells treated with UVA or singlet oxygen (14). Our results partly support this suggestion because the proportion of cases with elevated level of CD in the tumor tissue was higher in radiation-associated than in sporadic group. We therefore suppose that level of the CD in thyroid tumors may be associated with radiation exposure in an indirect way.

To further characterize large-scale mutations in mtDNA, we used a multiplex PCR with fluorescent primer to perform fine profiling of spectrum of structural mutations in mtDNA. This approach allows introduction of a quantitative measure of mtDNA alterations for each specimen. Data on the number of noncanonic bands were then subjected to statistical analysis against the parameters of mtDNA determined before.

It should be mentioned that PCR products of different molecular weight referred to in this work as mtDNA deletions may, in fact, originate from various types of structurally aberrant mtDNA molecules serving as PCR template, e.g., partially duplicated mtDNA, deletion dimers (or oligomers), and deletion monomers. Because question of structure of rearranged forms of mtDNA was not specifically addressed in our experiments, we used the term mtDNA deletions for simplicity, albeit it is strictly applicable only to deletion monomers.

Similar to results of the estimation of relative mtDNA level and content of the CD, elevated number of mtDNA deletions was found in most of tumor tissues of radiation-associated PTC cases. In FA, this index was somewhat lower, and in the group of sporadic PTCs, it accounted for less than a half of cases. Tendency for association of the number of mtDNA deletions with level of radioiodine contamination was only found in PTC tumor tissues. Latter observation taken together with the fact of greater prevalence of number of mtDNA deletions in same type of samples indirectly points to a possible association of these parameters with radiation exposure. This is more evidently supported by the finding of a moderate but significant dose dependency of the mtDNA deletion yield observed in irradiated primary thyrocytes.

Most striking was a highly significant positive correlation between the number of mtDNA deletions and mtDNA content in radiation-associated tumor tissues of PTC and FA but not in sporadic group. Noteworthy, this correlation was found between two parameters estimated by absolutely independent methods and its occurrence, at least in PTCs, was not because of the effect of outliers. By contrast, in normal tissue, the number of mtDNA deletions did not correlate significantly with mtDNA content and, furthermore, tended to decline in specimens with higher relative mtDNA content. Detailed analysis of the results demonstrate that there were 7 of 11 cases with coordinated elevation of both mtDNA level and number of mtDNA deletions in the tumor tissue in radiation-associated group of PTCs versus 2 of 9 of such cases in spontaneous PTCs. Although there is no statistically significant difference between these two groups, P = 0.092 by Fisher's exact test, strong bias of this index to the radiation-associated group is seen. The difference might reach the desired level of signif-
icance provided the number of cases under investigation was larger. Thus, we assume that synchronous elevation of the number of mtDNA deletions and mtDNA level in the tumor tissue may be attributable to radiation-associated PTC, whereas discordance of these two parameters is more characteristic to spontaneous cases. Another consideration is the opposite character of association of the number of mtDNA deletions with mtDNA level in tumor and normal tissue implying systems of mtDNA maintenance and replication may influence the formation and/or determine the life span of altered mtDNA molecules. If in tumor cells there might be some deficiency of such systems leading to mtDNA instability, in normal cells the preserved machinery can relatively efficiently repair or eliminate newly formed aberrant mtDNA molecules. As a result, in tumor cells an increased number of mtDNA molecules provides more abundant substrate for the mutational events and elevates the probability of the formation of aberrant mtDNA variants. In normal cells, as long as the specific rate of the formation of structurally mutated mtDNA molecules is not changed, relative content of mutated mtDNA variants would decline with the increase of total number of mtDNA molecules. This reasoning seems to fit better the radiation-associated group, suggesting that the exposure may comprise an additional driving force of the enhanced mtDNA mutagenesis in this type of thyroid tumors. As seen from experiments with primary cultures of human thyrocytes, a proportion of deletions in mtDNA can be induced by radiation in a dose-dependent manner. Taking into consideration that normal tissue or primary thyrocytes have some basal level of mtDNA molecules with deletions, it is plausible to speculate that total load of aberrant mtDNA species is composed of two parts. One is formed with a background rate determined by the tissue-specific mitochondrial microenvironment and another part may arise because of the effect of external mutagens such as radiation. Naturally forming mtDNA deletions may take place as a result of recombinational processes mediated by protein-mtDNA and protein-protein interactions around regulatory and functional regions of mtDNA (62–65), including some possible occasional errors in functioning of other factor(s) responsible for the maintenance of human mtDNA integrity. Recently discovered Twin-kle shown to be involved into the pathogenesis of autosomal-dominant progressive external ophthalmoplegia characterized by multiple mtDNA deletions may comprise one of such factors (66). Aberrant mtDNA variants were suggested to exist in a dynamic equilibrium when there is a surge of newly formed ones taking place in parallel with the elimination of already existing molecules (65). Eventually, it may be found that genotoxic agents lead to the formation of structurally mutated mtDNA molecules additional to those generated at the background rate (67, 68). However, questions as for their stability and relevance remain to be clarified.

Abundance of the aberrant mtDNA molecules detected in our experiments was extremely low as seen from the comparison with relative content of mtDNA with CD, which according to estimates, may be up to 0.1–4.3% of total mtDNA (25). From the molecular analysis of mtDNA in mitochondrial diseases, it is known that only threshold of aberrant mtDNA exceeding 60% of total number of mtDNA molecules would result in the development of a disease phenotype (Ref. 69 for review). Therefore, low abundant deletion-type mtDNA species observed in our series are unlikely to be causally involved into thyroid tumorigenesis but rather comprise a consequence of it and/or may reflect effects of mtDNA interaction with microenvironmental and macroenvironmental factors. Accordingly, the increase in the number of mtDNA copies observed in many tumors is not attributed to the propagation of aberrant mtDNA molecules but is rather evoked by unknown yet mechanism.

Sequencing of PCR products representative of aberrant mtDNA revealed the presence of homologous fragments around the break-points in most cases. The occurrence of mtDNA deletions with similar characteristics was described in aging, normal heart muscle, as well as in diseases such as inclusion body myositis and progressive external ophthalmoplegia (65, 70–73). Mechanism of the generation of such deletions was suggested to be slipped mispairing during the mtDNA replication, effect of reactive oxygen species promoting the DNA breaks, DNA repair in structurally dependent manner, and illegitimate mtDNA recombination (42, 65, 67, 71–76). In fact, structural likeness of sequences around the breakpoint may be suggestive of the formation of mtDNA deletions according to a mechanism similar to nonhomologous end joining observed in the nuclear DNA (77).

Absence of the correlation of the CD level with any other index and, most importantly, with number of mtDNA deletions implies that the formation of the CD and other deletion type mtDNA molecules are largely dissociated. Generation of the CD might be mostly determined by the structural identity of 13 bp fragments around the breakpoints, whereas random mtDNA deletions may arise as a result of an effect of intrinsic and external genotoxic factors. Supportive to this suggestion, the finding of Ray et al. (15) demonstrated that spectrum of mtDNA deletions, not just the CD, was a more adequate estimate of an overall DNA damage in skin.

In conclusion, our work demonstrated a strong correlation between the number of mtDNA deletions and level of mtDNA content in the tumor tissue of radiation-associated human PTC and FA, suggesting that evaluation of mtDNA status in thyroid tumors may be useful in attempts to elucidate molecular distinctive features of radiation-associated thyroid tumor diseases. We anticipate that the determination of not only one index but a combination of several parameters, including the mtDNA status, would allow more precise distinction between radiation-induced and sporadic human thyroid tumors.

REFERENCES


Large Deletions in Mitochondrial DNA in Radiation-associated Human Thyroid Tumors


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