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RADIATION RESEARCH **165**, 202–207 (2006)
0033-7587/06 $15.00

Mitochondrial DNA Mutations in Individuals Occupationally Exposed to Ionizing Radiation

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Wilding, C. S., Cadwell, K., Tawn, E. J., Relton, C. L., Taylor, G. A., Chinnery, P. F. and Turnbull, D. M. Mitochondrial DNA Mutations in Individuals Occupationally Exposed to Ionizing Radiation. ***Radiat. Res.*** **165, 202–207 (2006).**

Mutations in a 443-bp amplicon of the hypervariable region HVR1 of the D-loop of mitochondrial DNA (mtDNA) were quantified in DNA extracted from peripheral blood samples of 10 retired radiation workers who had accumulated external radiation doses of >0.9 Sv over the course of their working life and were compared to the levels of mutations in 10 control individuals matched for age and smoking status. The mutation rate in the 10 exposed individuals was 9.92×10^{-5} mutations/nucleotide, and for the controls it was 8.65×10^{-5} mutations/nucleotide, with a procedural error rate of 2.65×10^{-5} mutations/nucleotide. No increase in mtDNA mutations due to radiation exposure was detectable ($P = 0.640$). In contrast, chromosomal translocation frequencies, a validated radiobiological technique for retrospective dosimetric purposes, were significantly elevated in the exposed individuals. This suggests that mutations identified through sequencing of mtDNA in peripheral blood lymphocytes do not represent a promising genetic marker of DNA damage after low-dose or low-dose-rate exposures to ionizing radiation. There was an increase in singleton mutations above that attributable to procedural error in both exposed and control groups that is likely to reflect age-related somatic mutation.

INTRODUCTION

In addition to the nuclear genome, each human cell contains thousands of copies of a 16.5-kbp circular molecule of double-stranded DNA located in the mitochondria. This extrachromosomal genome codes for a small number of proteins involved in the respiratory chain (13 protein-coding genes) as well as two ribosomal RNA subunits and 22 tRNA genes. In addition, each mtDNA copy contains a short sequence, the D loop, involved in transcription and replication of mtDNA. Inherited defects in mtDNA may result in mitochondrial dysfunction that can contribute to degenerative disease (*1–3*). Such defects can affect all copies of the mtDNA within a cell (termed homoplasmy), or there may be a mixture of mutated and wild-type mtDNA copies (heteroplasmy). Due to the multi-copy nature of the mtDNA, with multiple mtDNA per mitochondrion and many mitochondria per cell, when defects exist in heteroplasmic form, a threshold value of mutant types must be surpassed before mutations cause a biochemical defect of the respiratory chain (*4*).

The mitochondrial genome is characterized by a higher spontaneous mutation rate than the nuclear genome (*5*). This is thought to be due to the absence of histones protecting the coiled DNA as well as the immense oxygen metabolism and presence of reactive oxygen species in the mitochondrion. In addition, the mitochondrion has an underdeveloped DNA repair repertoire compared with the nuclear genome (*6*). As a consequence, in addition to inherited mutations, sporadic somatic mutations can occur. Numerous point mutations and insertion or deletion of sequence (indels) have been characterized in mtDNA (*6*). Such sporadic mutations have been seen to accumulate with age in various tissues (*7*).

The tendency for cells to accumulate mtDNA mutations suggests that mtDNA might be a useful biomarker of radiation exposure. MtDNA is susceptible to genotoxic damage including that induced by exposure to γ radiation (*8*) and X radiation which has been shown to damage isolated mtDNA and cellular mtDNA in a dose-dependent manner (*9*). The frequency of the common deletion, a species of mtDNA with a 4977-bp deletion, has also been seen to increase in irradiated cell lines (*10*). This has led to the suggestion that the common deletion may prove useful in retrospective biological dosimetry (*11*). However, when the incidence of the common deletion was measured in individuals exposed by virtue of atomic weapons testing at Semipalatinsk, Kazakhstan and compared to unexposed populations there was no difference in frequency (*12*).

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While we recognize that ionizing radiation is known to be capable of inducing large-scale DNA deletions in the nuclear DNA (*13*), and deletions are inducible in mtDNA, radiation exposure is also associated with the introduction of point mutations (*14*). It is point mutations that are the target of the present study. mtDNA point mutations have been reported after acute *in vivo* exposure to high doses of ionizing radiation in individuals undergoing radiotherapy (*15*), and an increase in such mutations has been seen after chronic exposures to much lower dose rates of ionizing radiation in families inhabiting naturally radioactive areas (*16*). Since most copies of mtDNA carrying deletions are lost from circulating blood cells (*17*) while neutral point mutations appear not to be selected against, mtDNA point mutation frequencies rather than levels of large-scale deletions, such as the common deletion, may be a suitably sensitive biomarker of exposure to low doses of ionizing radiation such as those experienced in an occupational setting.

Selection appears to be weaker on mutations arising in non-coding regions of mtDNA (*18*), and consequently it is preferential to study such regions when quantifying mutation accumulation. The non-coding D-loop of the mtDNA has been shown previously to accumulate point mutations in studies of aging individuals (*7, 19*) and of those exposed to radiation (*15*). Here we examine the frequency of point mutations within the hypervariable region 1 (HVR1) of the mitochondrial DNA in a group of retired radiation workers exposed occupationally to ionizing radiation and compare them to point mutations in unexposed controls.

TABLE 1
Data on Age, Smoking and External Dose

Sample[a]	Age at sampling (years)	Smoking habit[b]	Dose (Sv)
Exp1	73	LXS	1.329
Exp2	72	N	1.143
Exp3	77	N	1.109
Exp4	68	HXS	1.015
Exp5	68	LS	0.946
Exp6	78	N	1.204
Exp7	79	HS	0.986
Exp8	76	LXS	1.873
Exp9	80	N	1.161
Exp10	67	N	0.920
Mean	73		1.169
C1	72	LXS	0.004
C2	71	N	0
C3	80	N	0.018
C4	68	HXS	0
C5	69	LXS	0.003
C6	73	N	0.006
C7	76	HXS	0.006
C8	74	LXS	0.016
C9	77	N	0
C10	69	N	0
Mean	72		0.005

[a] Experimental samples (Exp1–Exp10) and control samples (C1–C10).
[b] N = never smoker, LXS = low ex-smoker, HXS = high ex-smoker, LS = low smoker, HS = high smoker.

MATERIALS AND METHODS

Samples

All participants were retired employees of the British Nuclear Fuel Ltd (BNFL) Sellafield nuclear fuel facility. Samples were taken from 10 individuals who had received cumulative external radiation doses >0.9 Sv measured through film badge monitoring (*20*). Exposures occurred over a period of 20–37 years and were within the permitted limits operating at the time. Ten individuals who had received doses <0.02 Sv and who were matched for age and smoking status provided control samples. All participants were British Caucasians. Details of participants are given in Table 1.

Approval for the study was given by the West Cumbria Local Research Ethics Committee.

Molecular Methods

DNA was extracted from peripheral blood lymphocytes according to standard methods and standardized to 100 ng/μl. A 443-bp fragment of the HVR1 region of mtDNA was amplified with the primers L15995 (5′-CCA CCA TTA GCA CCC AAA G-3′) and H16401 (5′-GAT TTC ACG GAG GAT GGT G-3′). To minimize PCR-generated mutations, we used the thermostable proofreading polymerase *PfUUltra*, which has an extremely high fidelity (*21, 22*). PCR mixes contained 1× PCR buffer, 200 μ*M* dNTPs, 25 pmol of each primer, 100 ng DNA, and 2.5 U *PfUUltra* DNA polymerase (Stratagene). Amplification was undertaken on a PE9700 thermal cycler with conditions of 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min with a final extension time of 8 min. PCR products were purified using the StrataPrep® PCR purification kit (Stratagene) according to the manufacturer's instructions.

Purified PCR products were cloned into PCR-script™ Amp-SK(+) (Stratagene) and transformed into XL-10-Gold®. Recombinant plasmids were identified by blue-white color selection. After overnight growth of white colonies in 3 ml LB broth, plasmids were purified with a QIAprep® Spin Miniprep kit. Inserts were screened by agarose gel electrophoresis after *Hin*dIII-*Sac*I digestion. The fidelity of the assay was determined through sequencing of plasmids derived from amplification of a single clone. Plasmids from this experiment were standardized to 100 ng/μl and were then sequenced on a Beckman Coulter CEQ8000 with Quickstart sequencing reagents. Plasmid DNA containing inserts derived from experimental samples was standardized to a concentration of 100 ng/μl and then sent for commercial sequencing (MWG-Biotech).

Sequence Analysis

Sequences were aligned against the revised Cambridge reference sequence (CRS) (*23, 24*) using ClustalX (*25*). The 405 bp of sequence between positions 15996–16400 was successfully read from each copy. Discrepant positions were verified through manual comparison with the electropherogram.

Heteroplasmies were defined as mutations found in more than one clone of a single individual, and these were not included for the determination of mutation rate. This is a more conservative methodology than that applied by Simon *et al.* (*26*), who excluded only those mutations in fewer than three clones. Excluded mutations are listed but were not used in our estimation of mutation rate. Indels involving ≥2 bp were treated as a single event. Mutation rate was calculated as number of mutations/(number of clones sequenced × 405), where the length of the insert DNA without primers is 405 bp. Mutation levels in high-dose individuals and in controls were compared using Fisher's exact test.

RESULTS

Procedural Error Rate

Plasmid DNA from a single clone containing a PCR product (443 bp) of the HVR1 region of the mtDNA was used as template for PCR and the amplification product was cloned. Of 93 full-length clones sequenced, only one contained a mutation (T16301C). This suggests a procedural error rate of 2.65×10^{-5} mutations/nucleotide ($1/93 \times 405$ bp).

Sample Analysis

DNA isolated from peripheral blood samples was used as templates for amplification of a 443-bp section of the HVR1 region that was subsequently cloned. Up to 100 separate cloned amplicons were then sequenced. A small fraction of clones exhibited sequences with multiple substitutions (>30% sequence divergence) and numerous indel events. These displayed hallmarks of pseudogenic sequences and were discounted from further analysis. Nuclear pseudogene copies of mtDNA genes are recognized (*27*), and amplification of mitochondrial control region pseudogene sequences is recognized as a problem when PCR is undertaken on DNA isolated from blood samples (*28*).

Each of the 20 samples differed in homoplasmic sequence from the CRS by between 0–8 bp (Table 2). Each sample also exhibited mutations in individual mtDNA copies. Where two or more identical mutant copies were seen, these were counted not as two (or more) identical singleton substitutions but as a heteroplasmy (Table 2). Multiple heteroplasmy was identified in individual C4, with three different mtDNA types encountered (disregarding singleton substitutions). These were within the 16184–16193 poly-C tract known to be unstable during replication, which results in multiple poly-C tract length variants (*29*).

Singleton substitutions were seen in between one and eight of the clones examined from each individual (Table 2). While more substitutions were observed in the exposed samples, no significant increase in mutation rate was detected. The overall mutation rate based on singleton substitutions within the exposed samples was 9.92×10^{-5} mutations/nucleotide, and for the control samples it was 8.65×10^{-5} mutations/nucleotide. This is not significantly different ($P = 0.640$). Mutations identified by the sequencing were point mutations, deletions or insertions, with 70.6% transitions, 20.6% transversions, and 8.8% indels in the controls and 82.1% transitions, 10.3% transversions, and 7.7% indels in the exposed samples.

DISCUSSION

We have addressed the question of whether low doses of radiation received in an occupational setting resulted in a significant increase in mutations in the HVR1 region of mitochondrial DNA detected through cloning and sequencing of PCR amplicons. Gamma radiation produces a similar mutational spectrum to that resulting from oxidative damage (*30*), including mainly base substitutions, predominantly GC→AT transitions followed by GC→TA transversions, but also indels (*30*). The types of mutations identified in this study agree favorably with those expected of mtDNA, i.e. 80% transitions and 6% indels (*6*), although the control samples exhibited fewer transitions and more transversions. Although point mutations were detected, we found no increase in mutation levels when individuals exposed to up to 1.873 Sv (mean 1.169 Sv), in small fractionated doses over the working life of the individual, were compared to control individuals matched for age and smoking status and employed at the same nuclear facility who received less than 0.02 Sv radiation (mean 0.005 Sv).

Chromosomal translocations, a recognized and validated biomarker of exposure to ionizing radiation (*31*), have been measured previously in these individuals (*32*). When translocation frequencies are compared between these same individuals, there is a statistically significant difference that is attributable to radiation exposure (control group translocation frequency $= 17.41 \pm 2.25 \times 10^{-3}$ per genome equivalent; exposed group translocation frequency $= 28.41 \pm 2.87 \times 10^{-3}$ per genome equivalent; $P = 0.041$). This indicates that mtDNA is not as sensitive a biomarker of cumulative radiation exposure as the measurement of translocation frequencies.

DNA for this study was extracted from circulating peripheral blood lymphocytes, and it has been suggested that due to the high turnover of blood, accumulation of mtDNA mutations may be inhibited (*17*). However, since the cumulative exposures of the high-dose group were acquired over the total working life of the individuals, it is the exposure of the hematopoietic stem cells that is important, since circulating lymphocytes are short lived and could therefore provide information only on recent exposures. Mutations in the mtDNA have been demonstrated to accumulate in colonic stem cells (*33*), and we would therefore expect that if fractionated exposure to low doses of radiation did result in significant increases in mtDNA mutations, they would be demonstrable in the descendant lymphocytes with this assay. However, the individuals studied here ceased employment at the Sellafield nuclear facility 10–26 years previously, and thus it is possible that mtDNA mutations are not sufficiently persistent and that although mtDNA mutations were induced by exposure, the mutations acquired were subsequently lost. Although mtDNA mutations are recognized to accumulate with age (*7, 19*) and mutations within the D-loop are not believed to be under strong selection pressure (*18*), persistence of mutations has not been tested adequately. While chromosomal translocations are a stable biomarker of radiation exposure of hematopoietic stem cells (*34*), the kinetics of mitochondrial replication is radically different from that of the nuclear DNA (*1*), and the long-term persistence of mtDNA muta-

TABLE 2
Mitochondrial DNA Mutations Identified in the Experimental Group (Exp1–10) and the Control Group (C1–10)

DNA sample	No. clones sequenced[a]	Homoplasmic base changes compared to CRS[b]	Heteroplasmy[c]	Singleton substitutions[d]
C1	100	A16235G, C16261T, C16291T, C16292T	No	C16021G, G16034A, C16057T, C16107T, T16292C*, G16329&T16330 del§, G16336 del, C16355T
C2	96	C16069T, T16126C, G16145A, T16231C, C16261T	A16302G (11)	G16039A, G16047T, A16212T, C16188T, T16189C, C16348T
C3	98	C16261T, A16293G, T16311C	No	G16153A
C4	96	N/A	No	C16111T, T16131G, C16191T
C5	94	A16293G, T16311C	No	A16081G, A16180G
C6	97	T16263C	No	A16181C, C16258ins
C7	97	T16224C, T16311C	T16093C (6)	T16008C, C16056T, C16256T
C8	97	T16092C, T16140C, A16293G, T16311C	C16092T (3)*	A16305G, C16375T
C9	98	N/A	No	T16136A
C10	97	T16297C	No	G16084A, T16172C†, A16269G†, C16270T, C16291T, C16393A
Exp1	100	C16301T, A16343G, T16356C, G16390A	No	A16220G, A16317G, A16383G
Exp2	97	N/A	No	G16000A†, T16093C, T16093G, C16095T, C16328T, T16347C, G16390A†
Exp3	98	C16286T	No	C16107T, A16162 del, G16244A, T16311C
Exp4	96	T16189C	G ins‡ (13) GG ins‡ (10) T16189 del (11)	A16180 del, A16183C
Exp5	96	No	No	G16049T, A16146G, T16172C, C16365T
Exp6	97	T16093C, C16221T, C16266T, A16399G	C16093T (46)	T16020C, C16114T, A16207G
Exp7	97	G16129A, C16223T, G16391A	No	G16039 del, C16111T†, T16124C, G16125A, C16256T†
Exp8	97	C16069T, T16126C	No	G16129A†, C16176T, A16258T, C16287T, G16346A†
Exp9	96	T16093C, C16192T, A16207G, C16256T, C16270T, C16291T, T16324C, A16399G	C16093T (5)*	T16029C, T16192C*
Exp10	96	T16093C, T16224C, T16311C, G16319A	C16093T (10)*	A16293G, A16343G, G16390A, G16391A

[a] No. clones sequenced = numbers of full-length clones successfully sequenced.
[b] Homoplasmic base changes are given relative to the position in the Cambridge reference sequence (*23*, *24*).
[c] Heteroplasmies are given with numbers in parentheses indicating the number of clones displaying the heteroplasmic position (*indicates revision of sequence to the sequence of the CRS).
[d] Singleton substitutions are given as positions relative to CRS (*23*, *24*) († = substitutions found in same clone, § = 2-bp deletion counted as single event). ‡ = insertions in the 16184–16193 poly-C tract.

tions after historical exposure to genotoxic agents is unknown.

The finding that no significant increase in mtDNA mutations results from chronic occupational radiation exposure contrasts with the increase in mtDNA mutation frequency seen in patients exposed to therapeutic doses of radiation compared to nonexposed patients (*15*). However, the doses and dose delivery regimens differ markedly. Individuals undergoing radiotherapy in the study of Wardell *et al.* (*15*) were a mixed cancer group who received total doses between 35–152 Gy (to the target organ) in an acute fashion. No information is available about the dose received by the bone marrow and thus the dose received by the hematopoietic stem cells. In contrast, the retired radiation workers for this study received much lower doses in small daily increments. In a study of individuals inhabiting the area around Semipalatinsk, a nuclear testing site for the Soviet Union, no increase in the frequency of the common deletion was seen in comparison to controls (*12*). These individuals would similarly have received their accumulated dose in a chronic fashion. Since the common deletion is known to be inducible by ionizing radiation (*10*), this also suggests that low-dose radiation received chronically either does not result in mtDNA mutations (or is efficiently repaired) or that

the system employed in these studies is not sensitive enough to detect small increases with the sample sizes available. It is also interesting to note that studies of native voles or experimentally enclosed laboratory mice in the vicinity of Chernobyl have failed to detect an increase in mitochondrial mutations after chronic exposures (*35–37*).

We are mindful that this study employed only small sample sizes (although examining a large number of clones per individual). However, although an increase in sample size may be appropriate, the methodology employed is time consuming and expensive and does not lend itself to high throughput. To extend the sample size in the current study, it may be that a more appropriate technique for examining large numbers of clones from large numbers of samples would involve screening of inserts with denaturing high-performance liquid chromatography (*38*).

Although no increase in mutations of the mitochondrial control region attributable to radiation exposure was detectable, we did see an increase in mitochondrial mutations in both exposed and control populations that is above the procedural error rate of the system employed (i.e. mutation rate in the technical controls). This presumably reflects an increase in mitochondrial mutations due to age in these individuals (mean age 73 years, range 67–81, for exposed samples, and mean age 72 years, range 68–81, for controls). However, we have no young population with which to compare for the specific examination of age effects. The mutations we detect are, however, individually rare.

There is thus no evidence that occupational exposure to low doses of radiation results in an increase in mtDNA mutation rate in hematopoietic stem cells as measured through the examination of peripheral blood lymphocytes.

ACKNOWLEDGMENTS

We thank Mrs. Caroline Whitehouse for identifying the matched samples and for examining the data on translocation frequencies. This study was funded by British Nuclear Fuels Limited (BNFL).

Received: July 11, 2005; accepted: September 15, 2005

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