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Effect of Low Dose Ionising Radiation on the Amount of Mitochondrial Common Deletion and D-Loop Tandem Duplication in Human Peripheral Whole Blood

Abstract

The Hungarian Defence Forces assume a significant role in disaster prevention, including nuclear accident prevention tasks, so both the command and executive staff can stay in the higher-than-natural dose area. The current "gold standard" microscopic method aimed at determining the radiation dose suffered is the dicentric chromosome assay (DIC), although sensitive and accurate, it is very time-consuming. Monitoring changes in the amount of common deletion (CD) of mitochondrial DNA (mtDNA) and in the D-loop a tandem duplication (TD) of mtDNA may be a reliable marker of exposure to ionising radiation. This work used the PCR method to investigate how CD and TD change in human blood samples after X-ray irradiation. The CD appears to be a particularly useful marker, as its maximum is below the threshold for clinical symptoms. This work is the first to show the relationship between radiation and tandem duplication. The TD we investigated occurred more frequently in irradiated human blood samples than native ones. During the future development of the diagnostic tool, both CD and TD are informative and would be used together in a PCR system to detect acute and cumulative irradiation. In recent years, more and more health institutions are dealing with molecular biological diagnostic work. In a disaster situation, if the laboratory capacity of the Hungarian Defence Forces would not

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be sufficient for this, more external laboratories can be involved for PCR measurements than for traditional microscopic work.

Keywords: biodosimetry, radiation detection, mitochondrion, DNA, deletion

Introduction

Due to its function, the Hungarian Defence Forces must be prepared to carry out the tasks performed in the CBRN (chemical, biological, radiological, and nuclear) operational environment, or even for disaster prevention activities for example in the event of a nuclear emergency in the domestic environment.

One of the important tasks of disaster prevention is radiation protection. However, in the event of a disaster or an unexpected situation, the pre-planned protection cannot always be implemented, or it is only partially implemented, and since the Hungarian Forces assume a significant role in the tasks of disaster prevention, including nuclear accident prevention, both the command staff and the executive staff work in an environment with higher exposure rate than normal. This, of course, comes with the risk of suffering significant radiation dose.

Radiation exposure can also occur in connection with a known radiation event, but even in this case, we do not know exactly how much radiation dose each person received based on the field conditions and the time spent. In addition, for example, radiation sensitivity may differ from person to person.

In a disaster situation, such as a nuclear or workplace accident, or during a terrorist attack, those present may be exposed to radiation, and, in the event of a large-scale industrial accident, the radiation may reach human bodies for thousands of kilometres, i.e. beyond the borders of the affected country (for example, Chernobyl or Fukushima).

The visible symptoms of radiation exposure – skin reaction, vomiting, headache, diarrhoea – only appear with a delay of several hours, depending on the situation, in the case of a larger absorbed dose of more than 1 Gray. These are nonspecific and can easily be confused with symptoms of other diseases, so patients may not receive appropriate treatment if the doctor does not suspect radiation exposure (for example, in the case of Alexander Litvinenko).[4]

During an event that affects the masses, there will be many people among a large number of injured who may not even show clinical symptoms, but in their case, the risk of various cancers will increase significantly later on, and that must be taken into account that there will be those who develop symptoms, but they did not develop in connection with radiation exposure (stress, chemicals, trauma).

In the event of a mass casualty, the responsible commander must set up an emergency sequence among those involved, this process is called triage. For persons not wearing a physical dosimeter, the level of radiation exposure can only be estimated afterwards, with the help of various biodosimetry procedures.

[4] Harrison et al. 2017: 266–278.

Biological dosimetry is a procedure for assessing the radiation dose that affected the body based on the damage caused,[5] more precisely, based on the errors accumulated during the enzymatic repair of the damage in the affected but surviving cells. DNA repair mechanisms are immediately activated and take place in both the nucleus and mitochondria. Traces of repair persist throughout the cell's lifetime, such as deletions, duplications, and translocations. If the cell is capable to divide, the new cells get and give their successors changed DNA.

Biodosimetry provides an answer to whether radiation exposure has occurred, how much radiation those present suffered, and the resulting cell damage through laboratory examination of those involved. In this way, the injured person can receive appropriate medical care and legal remedies.

These methods can be used to determine the radiation sensitivity[6] of soldiers tasked with damage rescue, and by checking people returning home with complaints or with higher radiation exposure, appropriate therapy[7] can be selected in the case of knowledge of specific radiation injuries.

For a long time, dicentric chromosome analysis using lymphocytes was the only biological dosimetry method, and it is still the most widespread, so-called gold standard technique.[8]

There are many other biological endpoints, such as micronuclei, translocations, and aberrations in early condensed chromosomes.[9] In addition to military reasons, reliable biodosimetry is also needed in occupational radiation protection to confirm or exclude suspected low-dose radiation exposure. For this, more and more laboratories are looking for new targets for example mRNA and protein expression studies,[10] mitochondrial deletion[11] to detect the radiation dose suffered, and this is made possible by rapidly developing molecular biological methods.

Our goal is to find PCR-based biodosimetry methods with higher throughput than microscopic methods, with good specificity, which we could use as a pre-screening test of the DIC method in a mass accident situation. Our current research focuses on radiation-induced mitochondrial deletions and duplications.

Human mitochondrial DNA (mtDNA) is present in several copies in individual mitochondria, on average 2-10 copies may occur. Human mtDNA is a 16.6 kb circular double-stranded DNA molecule that encodes 13 essential polypeptides for the respiratory chain and a set of RNAs (2 rRNAs and 22 tRNAs) for intramitochondrial translation.[12]

Among these tightly packed genes there is a 1.1 kb non-coding DNA region, called displacement loop (D-loop), which contains sequences important for the initiation of mitochondrial replication and transcription.[13] The location of the D-loop is at site

[5] International Atomic Energy Agency 2011.
[6] Kiss et al. 2013: 104–112.
[7] Dainiak et al. 2003: 473–496.
[8] Voisin 2015: 115–122.
[9] Deli 2018: 179–192.
[10] Schüle et al. 2022.
[11] Schilling-Tóth et al. 2011: 33–39.
[12] Anderson et al. 1981: 457–465.
[13] Clayton 1991: 453–478.

16024-576 bp according to the Cambridge reference sequence.[14] The structure consists of one double and one single DNA strand. The reason for this is that the complementary heavy strand starts to synthesise on the light chain, the process separates the original heavy chain forming a loop, hence the name of the region. Synthesis stops at the end of the D-loop, and the mtDNA becomes quiescent, and stabilising proteins bind to the single-stranded DNA stretch.[15] The duplications described in the literature develop in this area.[16]

Many mtDNA deletions are readily detected in non-dividing tissues such as the brain and muscle but are rarely detected in relatively rapidly dividing cells (such as white blood cells). The most commonly reported deletion is a 4977 bp deletion or "common deletion" (CD), originally observed in patients with mitochondrial myopathy.[17]

Materials and methods

Blood samples were collected with a venous puncture in a blood collection vacuum tube with sodium citrate. The samples were aliquoted into 1.5 ml microcentrifuge tubes according to the doses to be irradiated, during which 1.4 ml of blood was added to the tubes under atmospheric oxygen. Contact of blood with oxygen was avoided as much as possible to preserve its venous character since the free radical formation of mitochondria can be influenced by the amount of oxygen.[18] The colour of blood is the indicator of blood oxygenation. The samples were stored at room temperature until the irradiation.

Irradiation took place 2 hours after blood collection, samples were irradiated at room temperature using a Precision X-Rad 225 XLi X-ray system. The dose rates, distances and filters are indicated in Table 1. The doses were checked with a dosimeter. Irradiation was carried out at room temperature. After irradiation, the blood samples were incubated for 3, 24, or 48 hours at room temperature, protected from light.

Table 1: The dose rates, distances, and filters used

Dose	Copper filter (mm)	Distance (cm)	Time	Dose rate
0.05	6	125	3 min 18 sec	0.015 Gy/min
0.1	1	125	1 min 4 sec	0.094 Gy/min
0.5	1	60	1 min 14 sec	0.41 Gy/min
1	1	60	2 min 27 sec	0.41 Gy/min
2	1	35	1 min 38 sec	1.23 Gy/min

Source: compiled by the authors

[14] Clayton 1991: 453–478.
[15] Jiang et al. 2021.
[16] Damas 2014: 1261–1268.
[17] Wallace 1992: 628–632.
[18] Pesznyák–Sáfrány 2016: 108–110.

DNA isolation

Nucleic acid isolation was performed 3, 24, and 48 hours after irradiation using the QIAamp DNA Blood Mini Kit (Qiagen), according to the manufacturer's recommendation. The total DNA extract, containing both nuclear and mtDNA was used for polymerase chain reaction (PCR) analysis without further purification.

Primers used in this study

"CD": To detect the mitochondrial common deletion, the primer pair was designed for the two ends of the 4977 bp long deletion. Due to the short cycle time, this section cannot multiply in the wild type,[19] but it can after the formation of the deletion, so we were able to measure the amount of deleted DNA. Sequence of CD Forward primer: CCCACTGTAAAGCTAACTTAGCATTAACC,[20] sequence of CD Reverse primer: AGGTTGACCTGTTAGGGTGAGA.[21]

"TD": A back-to-back primer pair was designed for the detection of tandem duplication in the D loop of mitochondrial DNA, with 9 bases between the 5' ends of the primer pair. On the non-duplicated sequence, DNA synthesis would be initialised in the opposite direction, however, only if the duplication is present and the number of primary binding sites is also duplicated, product formation is experienced during the PCR reaction. Sequence of TD Forward primer: TTTTTGGCGGTATGCACTTTTAAC, sequence of TD Reverse primer: GAAATCTGGTTAGGCTGGTG. We designed this primer pair in such a way that they recognise and give products in case of several known duplications.

"mtDNA": replicates a section of the "minor arc" of the mitochondrial genome, which is present in both normal and deleted DNA (deletions rarely occur in this section).[22] This allows us to check the amount of all mitochondrial DNA. Sequence of mtDNA Forward primer: CTAAATAGCCCACACGTTCCC, sequence of mtDNA Reverse primer: AGAGCTCCCGTGAGTGGTTA.[23]

"GAPDH": refers to the amount of DNA (nuclear or nDNA) in the nucleus, which is proportional to the number of cells. Sequence of GAPDH Forward primer: CGACCACTTTGTCAAGCTCA, sequence of GAPDH Reverse primer: AGGGGTCTACATGGCAACTG.[24]

[19] Rogounovitch et al. 2002: 7031–7041.
[20] Rogounovitch et al. 2002: 7031–7041.
[21] Schilling-Tóth 2015: 52.
[22] Phillips 2014.
[23] Phillips 2014.
[24] Schilling-Tóth et al. 2011: 33–39.

PCR

During the PCR reaction, we used Quantinova master mix (Qiagen, Germany) containing SYBR Green intercalating dye, the measurements were performed on a BioRad CFX96 Real-Time PCR (BioRad, USA) device. With the primers described above, the relative amount of GAPDH, Minor and CD sequences were determined. After denaturation at 95 °C for 2 min, the reaction mixture was cycled 50 times at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, finally extended at 72 °C for 10 min. Melting curves were detected as the first qualitative check.

Gel electrophoresis

The PCR products were checked by 1% agarose gel electrophoresis. The gels were then evaluated using an Alpha Innotech Fluorchem 5500 Gel Imaging System and were detected fluorographically under UV light transillumination after staining with ethidium bromide. During the evaluation of CD, only those samples were considered/included, where the expected 374 bp product appeared during gel electrophoresis. The samples with inappropriate products were excluded from the data analysis. Since the used primer pair may amplify many other types of deletions, we also exclude samples where other products appear alongside the expected product, and the unexpected product is more pronounced.

In the case of tandem duplication, the back-to-back primers are located in opposite directions. The primers can bind even in the absence of duplication, but no double-stranded product is formed. Samples with no specific product show a smeared line in the gel. If the duplication is present, each primer can bind to 2 places on the mtDNA molecule. That is, in the case of tandem duplication, one more binding site is formed for both primers, so the reverse and forward pairs will be opposite each other, so in such a case, the duplicated section is multiplied exponentially. When checked by gel electrophoresis in the case of the simultaneous presence of duplication (and even triplication), one (or two in the case of triplication) band is formed.

Statistical analysis

Analysis of CD: The experimental data were collected with the program of the BioRad CFX 96 instrument. Data were then arranged with MS Excel.

The number ratio of the nuclei, mitochondria, and deletion mitochondria per unit volume was calculated from the measured Ct values of GAPDH (nucleus), mtDNA (minor arc), and CD (deletion marker) samples using the formula 1/(2Ct).[25] The experimental data processed during the statistical analysis are presented in mean ± SD format per dose. Two parallel measurements were made from the

[25] Livak–Schmittgen 2001: 402–408.

samples of the 7 donors for each dose. Measurement points were excluded due to the formation of an inappropriate product (according to gel electrophoresis) resulting in a different number of measurement results being associated with each point. At the points with several measurement results, the average of the points was assigned to the given donor. An exception to this is the normality test performed during the statistical control, where the points were considered separately. The deletion rate of the 4977 bp mtDNA was investigated by one-way analysis of variance. All statistical analyses were performed using ratio-paired t-tests with GraphPad Prism6 statistical software. $P < 0.05$ was considered to indicate a statistically significant difference.

Analysis of TD: The real-time PCR curve and the melting curve are difficult to interpret due to the non-specific "smear" products produced by the unusually oriented primers.

With the PCR device, all duplications are amplified and detected, which includes the region 376-427 (almost all known duplications). We did not make any distinctions in this series of measurements. All definite bands were counted in the gels run from the PCR samples made with the intercalating dye. This amount is plotted as a function of the applied doses.

Results

Changes in the number of common deletions 3 hours after X-ray irradiation of blood samples

For the timing of the examination of the CD marker, 3 hours from the irradiation seemed promising, when the potential synthesis of genomic and mitochondrial DNA should be considered less. We observed that at this short time, there were no significant changes in Ct values for GAPDH (nuclear marker), and mtDNA (mitochondrial marker).

The amount of deleted mitochondrial DNA changes sensitively as a result of irradiation, we can observe that it reacts more sensitively to radiation exposure than the mtDNA marker. In our tests, we took into account the CD/mtDNA ratio.

Regarding to the CD marker, after the increase at low doses, a very steep drop is visible, which can be observed at about 1 Gy, see Figure 1.

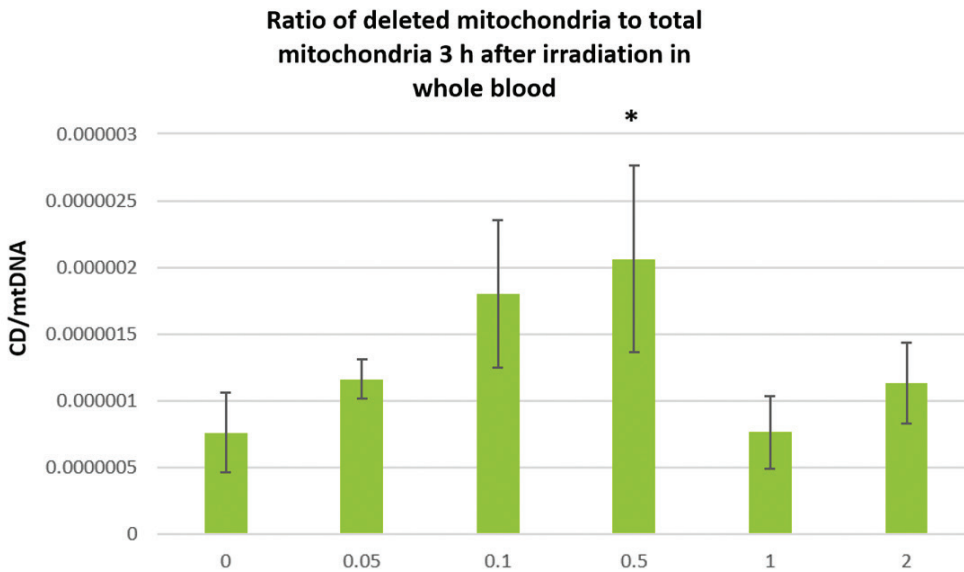

Figure 1: Quantitative change in the proportion of deleted and healthy mtDNA as a function of the irradiated dose after 3 hours (7 donors, mean, S.E.M.)

Note: An increase is observed between 0-0.5 Gy, which drops back to the baseline level at 1 Gy. (, P < 0.05, Ratio paired t-test)*

Source: compiled by Gábor Deli

We also performed the 12- and 24-hour incubations as mentioned in the methodology section, the course of the curves resembled the one presented above (see Figure 1), but the standard deviation was larger, and we did not obtain significant results.

Tandem duplication

Because of our previous experience with CD, the presence of D-loop tandem duplication was investigated 3 hours after X-ray irradiation, at 0-2 Gy dose. In the dose and time range used, the relative cell count remained unchanged, as we expected.

We counted the distinct bands in the gels run from the PCR samples made with the intercalating dye. The tendency can be observed that duplications are more likely to occur as a function of increasing irradiation dose, see Figure 2. The primer pair designed by us can theoretically amplify several known duplications.[26] Knowing the base sequence of the duplications, the method still needs to be refined by designing additional primer pairs and fluorescent probes.

[26] Damas 2014: 1261–1268.

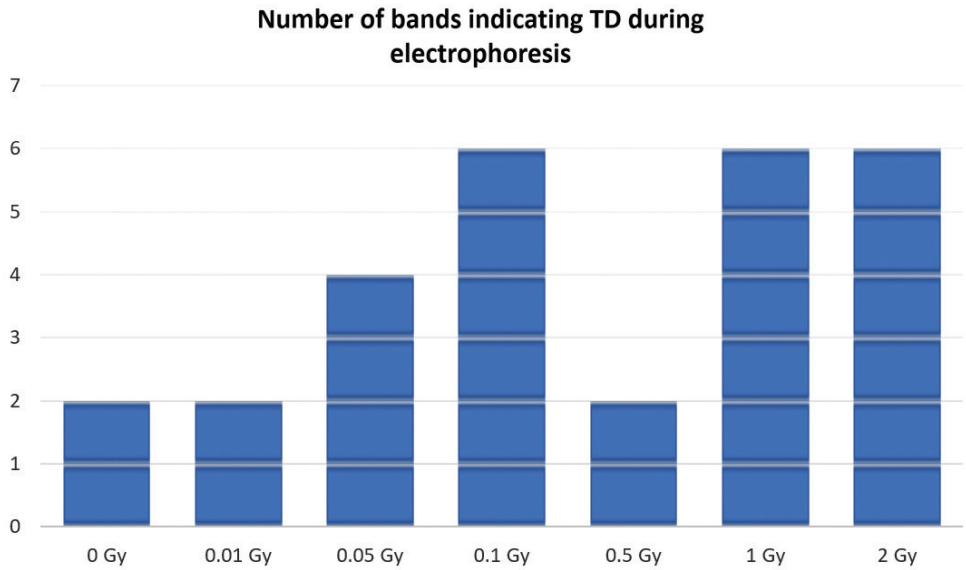

Figure 2: Duplication is rarer than CD. During the gel electrophoresis, the appearance of the bands was detected, and their sum was plotted (n = 7)

Source: compiled by Gábor Deli

Discussion

Radiation not only damages nuclear DNA but also mtDNA. After this, repair takes place in the mitochondrion, and the mtDNA resumes its circular form to avoid degradation.[27] Pieces can be lost during ring formation, less often they can be integrated, and deletions or insertions can occur.

One of these is the common deletion (CD) of mitochondrial DNA, a deletion of 4977 bp. The CD is formed in response to radiation and disappears rapidly in in vitro culture,[28] but Borghini et al. showed that the mtDNA 4977 deletion is significantly high in doctors who regularly catheterise, indicating that lifelong accumulation occurs in vivo at low doses.[29]

There are 2 identical 13 bp sequences in the mitochondrion beginning at sites 8471 and 13448 of mtDNA.[30] These two sequences are complementary, and if a double-strand break occurs anywhere in the section between them, a common deletion can occur first by pairing and then by DNA degradation. This is called the "slippage-replication" mechanism[31] and explains why random DNA damage anywhere

[27] Liao et al. 2022.
[28] Wanga et al. 2007: 433–442.
[29] Borghini et al. 2019: 976–984.
[30] Schon et al. 1989: 346–349; Yen 1991.
[31] Fontana–Gahlon 2020: 11244–11258; Shoffner et al. 1989: 7952–7956.

between the two stretches can cause such a specific deletion. The origin of synthesis of the heavy (57-441) and light chain (5721-5798) and promoter regions are not lost by the resulting degradation. Due to its unique formation mechanism, the "common deletion" is easily identified and is a very sensitive marker of mtDNA damage.

Since all genes in mtDNA are essential for the biogenesis and bioenergetic functioning of mitochondria, any mutations that lead to altered expression of these genes are expected to cause disturbances in energy metabolism and increased production of ROS released as intermediates during oxidative phosphorylation.[32] In addition to mitochondrial degenerative diseases, lifelong accumulation of mtDNA mutations and deletions is also believed to contribute to the aging process.[33] This is supported by the age-related decrease in oxidative phosphorylation and the accumulation of mtDNA mutations and deletions. As a marker of these processes, both aging and mitochondrial degenerative diseases show the accumulation of mtDNA common deletions.[34]

Studies have shown that this deletion can be used as a marker of oxidative damage to mtDNA even after very low doses of damage, as the lesion is amplified during mtDNA replication.[35]

There have been several reports that the CD form is not detectable to the same extent in whole blood.[36] Most authors use cell lines or primary tissue preparations to detect CD form after ionising radiation,[37] blood is rarely used for this purpose.[38]

Despite this, we chose whole blood, as blood is one of the most easily accessible tissues, and traditional biodosimetry tests also use blood. We also have to be very careful when taking samples, because the oxygen in the atmosphere and the oxygen bound in haemoglobin can change the tendency of DNA to break. Cell division could interfere with the effect of radiation, so we tried to minimise the time.

It was found that 3 hours after X-ray treatment, the CD increase varies according to a bell curve depending on the irradiation intensity, with the maximum observed around 0.5 Gy. The course of the curve showing the maximum of the CD/Min results is reminiscent of the course of the curve obtained by Schilling et al.[39] (on cell culture, but with a similar experimental set-up), which indicates that we are probably faced with a general phenomenon. The probable explanation for this phenomenon can be at a low dose interval that the higher the dose that hits the cell, the more breaks occur, and thus more deletions can form during the repair. However, by increasing the dose, the chance of mtDNA breaking both at the deletion site and outside of it increases, the mtDNA splits into two parts, and even during the formation of two smaller rings, no CD form is created.

[32] Kim et al. 2006: 10377–10383; Leach et al. 2001: 3894–3901.
[33] Wallace 1992: 628–632, Yen 1991; Jessie et al. 2001: 169–174.
[34] Cortopassi–Arnheim 1990: 6927–6933; Cortopassi et al. 1992: 7370–7374; Corral-Debrinski 1992: 169–180; Kaneko et al. 2012: 26–30; Lee et al. 2001: 67–74; Gerhard et al. 2002: 155–166.
[35] Ames 1989: 41–46; Fraga et al. 1990: 4533–4537.
[36] Lee et al. 1994a: 37–43; Ahmadi 2019: 250–254; Wang et al. 2013: 990–993.
[37] Schilling-Tóth et al. 2011: 33–39; Rogounovitch et al. 2002: 7031–7041.
[38] Borghini et al. 2019: 976–984.
[39] Schilling-Tóth et al. 2011: 33–39.

We observed that at this relatively short, 3 hours' time both the GAPDH and the mtDNA marker showed uniform, unchanged profiles, with a smaller standard deviation than after 24 hours.

We assumed, and our experiments seem to support, that because of radiation, not only deletion but also duplication can occur in mtDNA. The relationship between the occurrence of mitochondrial tandem duplications and ionising radiation has not yet been investigated. The D-loop is a partially duplicated section of mtDNA. If the two helices are broken at the same time because of radiation, the strands can be exchanged during the repair, and this can result in tandem duplication.

DIC is the gold standard method for biodosimetry.[40] It can be assumed that mitochondrial tandem duplicates are also radiation-specific aberrations, as are dicentric chromosomes since their formations show similar characteristics. According to the way of formation, it is understandable why and how specific the DIC formula is for ionising radiation.[41]

As a result of radiation, fractures do not occur randomly, but strictly along a straight line. In contrast, treatment with radiomimetic compounds (nitrogen mustard, 5-fluorouracil, methotrexate) usually results in random fractures.[42] Admittedly, bleomycin is an exception because one molecule can induce multiple breaks.[43] The condition for the creation of DIC is the simultaneous interruption of two DNA double helixes (four sugar-phosphate strands) that are at an adjacent location. Ionising radiation travels through tissue in no time, producing short-lived reactive radicals in its path that create side-by-side DNA double-strand breaks, and leaving other areas intact, keeping the cell alive in most cases. The formation of DIC during enzymatic repair is an exchange between the pieces of two broken double helixes, in such a way that the chromosome fragments containing the two centromeres are united. A cell containing a DIC is usually no longer capable of further division.

Translocations are radiation specific as well, they are formed in the same way as DIC, but the centromeric part is not transferred from one chromosome to another. They can be detected by fluorescent in situ hybridisation (FISH). As the further division of the cell is not hindered, so translocations persist for a long time, and the baseline is showing a significant increase with age.[44]

The tandem duplication in the D loop may therefore be promising for biodosimetry purposes if it becomes detectable in a PCR test system similar to that used for "CD". Since no genetic information is lost, we assume that loop D duplications can be detected longer than CD.

Lee et al. already observed in the early 1990s two tandem duplications (one 260 and one 200 bp) in the mitochondria of the muscles of elderly people.[45] The 200 bp repeat was found to be age dependent. Others have also observed that it

[40] International Atomic Energy Agency 2011.
[41] Hoffmann–Schmitz-Feuerhake 1999: 113–133.
[42] Deli 2022: 101–115.
[43] Deli 2023: 57–72.
[44] Whitehouse et al. 2005: 139–145; Sigurdson et al. 2008: 112–121.
[45] Lee et al. 1994b: 79–83.

often occurs in conjunction with common deletion.[46] We assume that it is difficult to separate the effects of cumulative doses of radiation and age since radiation affects our bodies throughout our lives. The 44 duplications described so far are at least partly in the area of the D-loop.[47]

This work of ours is a pioneering attempt to explore the relationship between radiation and TD. During our measurements, in the dose range, we used (0-2 Gy), the probability of the appearance of duplications also increased with the increase of the applied radiation dose. Regarding TD, PCR results are visible only after gel electrophoresis, although the use of fluorescent probes can increase the specificity of the method.

A product with a length of approximately 190 bp most often appeared in the gel as a result of X-ray radiation. This may correspond to the second most frequent duplication in the 200 bp long list compiled by Krishnan et al.,[48] and since there are 9 bases between our two designed primers, therefore the product will be shorter by that much.

The primer pair we designed can theoretically amplify 43 of 44 known duplications,[49] for the sake of clarification, we plan to check later by sequencing which one is the most frequent to collect information for the design of radiation-specific primers and probes.

The duplicated mtDNA can divide regularly and is not degraded, so there is a possibility of accumulation during life. Therefore, it is not surprising that the duplication can also appear in unirradiated samples.

After appropriate refinement of the method in the future – with NGS sequencing to a PCR probe system – we can distinguish the duplication variants, and by applying the TD together with the CD test, an informative PCR test can be produced that can also be used in radiation biology.

If both CD and TD will be detected simultaneously, the acute and cumulative effects of radiation could be detected.

Using this PCR test on the radiation effect as a pre-screening on-site examination reduces the number of people to be examined with microscopic methods, so it can help with triage and deciding on the appropriate treatment in disaster situations affecting many people.

In light of all this, measuring mitochondrial deletion and duplication can be very important from a disaster situation handling point of view.

The necessary infrastructure and the operating molecular biologists are present in several institutions of the country, in a disaster situation it is easier to involve external laboratories and find help for PCR measurements than in the case of difficult microscopic work.

[46] Krishnan–Birch-Machin 2006: 408–415.
[47] Damas 2014: 1261–1268.
[48] Krishnan–Birch-Machin 2006: 408–415.
[49] Damas 2014: 1261–1268.

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