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Experimental Detection of Mitochondrial DNA Insertions in Nuclear Genome of Chicken Embryos Developed from X-Ray Irradiated Eggs

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Abstract

The transfer of mitochondrial DNA (mtDNA) into the nuclear genome is a dynamic process, resulting in the formation of nuclear mitochondrial (numt) pseudogenes or numt-insertions. Experimental determination of *de novo* numt-insertions is limited by the extensive homology of mtDNA in the nuclear DNA (nDNA) of eukaryotes. Since chicken nDNA contains only 13 numt-pseudogenes, we tried to follow experimentally the induction of numt-insertions *de novo* in the nDNA of chicken (*Gallus gallus*) embryos developed from eggs subjected to X-ray irradiation. NDNA of chicken embryo liver were twice purified from free mtDNA by gel-electrophoresis and monitored by PCR. PCR were run to determine the numt-insertions in the nDNA of surviving embryos, using 11 primer pairs flanking regions of mtDNA size of 300-400 bp. However, the PCR of control group nDNA, by using the given primers, revealed no homology with mtDNA. PCR of nDNA of embryos from irradiated eggs testified the origination of amplified mtDNA regions in two among eight embryos. Two and three loci of mtDNA were reproducibly identified in purified nDNA from two individual embryos. The sequencing of PCR amplicons synthesized from these nDNA matrices showed that they were identical to mtDNA. Thus the results indicate that ionizing radiation can induce integration of mtDNA fragments into the nuclear genome, perhaps in the process of repair of double strand breaks in nDNA via a non-homologous end-joining mechanism. However, it can be assumed that the insertion of large fragments of mtDNA in nuclear genome, as in this experiment, is a rare event.

Keywords

Induction numt-insertions, Ionizing radiation, Chicken embryos

Introduction

The transfer of mitochondrial genetic material into the nucleus and its integration in the nuclear genome is commonly believed to be a continuous and dynamic process. Fragments of mitochondrial DNA (mtDNA) in the nuclear genome are found as non-coding sequences, known as nuclear mitochondrial (numt) pseudogenes or numt-insertions [1-6]. The localization of numt-pseudogenes in the nuclear genome is currently studied in many higher organisms from yeasts to humans. The numt-pseudogenes are distributed over different chromosomes, persist in the genome as "fossil molecular elements" and form a "library" of mtDNA fragments that have migrated into the nuclear genome, thus providing a highly significant information on the history of genome evolution [2,4-6]. Numt-insertions can be not only considered as neutral polymorphic sites but are often associated with carcinogenesis, aging and genetic diseases in humans [7-12].

The incorporation of mtDNA fragments into the nuclear genome requires their escape from mitochondria. This may occur due to mtDNA damage, destruction of mitochondria, or in the process of division and mitophagy of these organelles [2,13-15]. Blanchard and Schmidt [16] hypothesized that mtDNA fragments can integrate into the nuclear genome during the reunion of broken chromosomal ends. This assumption was supported by a number of studies, giving grounds for a possibility of repair of double-strand breaks (DSB) of nuclear DNA (nDNA) accompanied by "capturing" of mtDNA fragments through non-homologous end-joining (NHEJ), and also with participation of microhomology regions on terminal sequences [5,17-21]. These points to the dependence of *de novo* numt-pseudogene formation frequency on the rate of occurrence of DSBs in nDNA, the activity of their repair by the NHEJ mechanism, as well as on the amount of mtDNA fragments migrating into the nucleus from organelles. We have previously proposed that such events are likely to occur following the influence of ionizing radiation on the organism, which induces the damage of mtDNA and nDNA with formation of DSB [21]. However, the existent literature on this subject lacks experimental confirmation of numt-pseudogene formation *de novo* in the genomes of organisms subjected to ionizing radiation. Experimental identification of numt-insertions *de novo* is difficult because of numerous regions of mtDNA homology constitutively present in eukaryotic nuclear genomes. We made an attempt of experimental detection of *de novo* origination of extensive numt-insertions in the nDNA of chicken (*Gallus gallus*) embryo liver obtained from X-ray irradiated eggs. The realization of analogous experiments using mouse models or human cells has been unsuccessful because of the high density of numt-pseudogenes in their nuclear genomes. Our choice of chicken as an object for study was dictated by that the chicken genome originally contains only 13 numt-insertions (0.0001% of the genome size) [5,22], while mouse

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and human genomes have 190 and 871 numt insertions, respectively [5]. We show that numt-pseudogenes arise *de novo* in the nuclear genome of chicken embryos obtained from X-ray irradiated eggs.

Materials and Methods

Chicken eggs and their irradiation

Fertilized eggs from chicken (*Gallus gallus domesticus*) of the White Leghorn chicken breed were obtained from a poultry farm (Tula, Russia). Two days after laying, the eggs were placed in plastic containers (5 eggs in each) and subjected to X-ray radiation. Irradiation was carried out using an X-ray unit TU-12 ("Medrent", Russia) at 280 kV, 18 mA, with a dose rate of 1 Gy/min. The absorbed dose was 5 Gy. Immediately after irradiation, the eggs were put for incubation (two groups of 10 irradiated and control eggs) at standard temperature and humidity. After 19 days of incubation, the eggs were opened. The irradiated group had 8 live embryos, while all the10 embryos survived in the control group.

Isolation and purification of nuclear DNA

Liver samples were taken from embryos, cleaned from membranes and homogenized in buffer A (10 mmol/l Tris-HCl, pH 8.0, 0.5 mol/l sucrose, 25 mmol/l KCl, 10 mmol/l $MgCl_2$, 2.5% NP-40) at the ratio 1:3 at 2-3°C in a Dounce homogenizer. The obtained homogenate was 3-fold diluted with buffer A, filtered through a capron mesh and centrifuged at 1000xg for 15 min. The nuclei-containing sediment was washed with buffer A by resuspending and centrifuging. After purification, the nuclei were resuspended in lysis buffer (10 mmol/l Tris-HCl, pH 8.0, 1mmol/l EDTA, 0.5% SDS, 20mkg/ml RNase) and incubated at 37°C for 45 min. DNA was subsequently isolated using a standard phenol-chloroform technique. The obtained samples were purified by gel-electrophoresis in 0.7% agarose to remove free mtDNA. Following the first electrophoresis, agarose strips containing high molecular nDNA were cut out from the gel and purified by electrophoresis for the second time. Areas of agarose gel containing high molecular DNA were visualized with ethidium bromide using a UV transilluminator. DNA was extracted from agarose gel and its subsequent purification was done with "DNA Cleanup" (Bio-Rad Laboratories, Hercules, USA) according to recommendations of the manufacturer. The amount of DNA was measured by reaction with Pico Green, according to the manufacturer's protocol (Molecular Probes, Eugene, OR, USA) and fluorescence was registrated using Tecan Infinite 200 (Austria).

PCR of long DNA fragments (long-extension PCR)

PCR of long fragments (long-extension PCR) was carried out with DNA samples (the first fraction was isolated from nuclei and electrophoretically purified fractions) to evaluate the impurities of free mtDNA. An mtDNA region of 15495 bp (covering more than 92% of the mitochondrial genome) was amplified and flanked by primers H1255 (5'-CATCTTGGCATCTTCAGTGCC-3') and L16750 (5'-AGGACTACGGCTTGAAAAGC-3') used in [23]. PCR of a part of the gene *β-actin* (3193 bp) with primers For 5'-ACAATGGCTCCGGTATGTGC AA-3', Rev 5'-CTGTAAAGCCTTCATTCACATCTAT-3' [24] was used as a positive control for PCR with nDNA. PCR of long mtDNA fragments and the *β*-actin-coding gene was realized in the same tube. The PCR reaction mixture (a total volume of 25 μl) contained: 75 mM/l Tris-HCI, pH 8.8, 20 mM/l $(NH4)_2SO_4$, 2.5 mM/l $MgCI_2$, 200 μM/l of each dNTP, 250 nM/l of each primer, 0.01% Tween-20. The reaction mixture was supplemented with 5 ng of DNA and 1.5 U of a mixture of *Taq* and *Pfu* DNA polymerases (Thermo Scientific, USA). The enzyme mixture was added for the "hot start" after the initial denaturation of the DNA template at 94°C for 4 min. PCR was run for 40 cycles of: 1 min denaturation at 94°C, 30 s annealing at 64°C and 5 min elongation at 72°C, followed by the final 10 min incubation at 72°C. PCR products were run in 0.8% agarose gel. Synthesis of all primers, including those listed in Table 1, was done by "Syntol" (Moscow, Russia). In all cases, the PCR was run on a programmed thermal cycler "Tertsik" ("DNA Technology", Moscow, Russia). PCR products were visualized on a UV transilluminator following gel electrophoresis with ethidium bromide.

Table 1: Primers used in PCR for determination of mtDNA insertions in the nuclear genome of chicken embryo liver.

№ and name of primers	Sequence 5'→3'	mtDNA region (bp)
1./ DL F DLR	CAGCAACCCCTGCCTGTAATG GGTGGAAGAACCATAACCAAATGC	429-826
2./ Cyt b F Cyt b R	CCCCATCCAACATCTCTGCTTG ACAAAGGCGGTGGCTATGAGTG	14963-15260
3./ ND5a F ND5a R	GGCAACCTCGCTCTAATAGGAAC CAGCAGTTTTTGTGATGGTGGG	14196-14517
4./ ND5b F ND5b R	AGCAATCCGTTGGTCTTAGGAAC GCGATGAGGAAGGTGAGTAGGTAG	13016-13430
5./ ND4 F ND4 R	ATCATACTCTTGCCCACAGCCC GCTAAGTCGTTCTGGTTGGTTTCC	11510-11894
6./ COIII F COIII R	CCAACAGGAGTCAAACCCCTAAA AGTATCAGGCTGCTGCTTCAAATC	10274-10623
7./ ATP F ATP R	CAGGAGTGTTTTACGGACAATGC TTCAGGGGGTGGGTTTAGTTG	8893-9212
8./ COI F COI R	GCCTAACGCTTCAACACTCAGC AAGGGGGGTAAACTGTCCATCCTG	6613-7041
9./ ND2 F ND2 R	CCGAGCGATTGAAGCCACTATC CTAAATGGGAGATGGATGAGAAGG	5390-5779
10./16S F 16S R	AAAAGAACACAACCTCCTCCAGC GCAGGCATCACCTTCAATACTTG	2842-3194
11./12S F 12S R	CAGGGTTGGTAAATCTTGTGCC CGTTTGTGCTCGTAGTTCTCAGG	1523-1819

PCR of mtDNA sequences using nDNA template

PCR was carried out using purified nDNA samples isolated from liver from both groups of embryos. A total of 11 pairs of primers were used, which corresponded to mtDNA and the flanking regions of 300–400 bp (Table). These primers were taken from [25,26]. The reaction mixture (25 μl), including primers and purified nDNA (5 ng), contained the same components, which were used for PCR of long fragments of mtDNA. PCR was run for 40 cycles: 30s denaturation at 94°C, 30s annealing at 62°C and 30s elongation at 72°C, followed by the final 4 min incubation at 72°C. The products of PCR amplification were analyzed electrophoretically in 1% agarose gel.

Sequencing PCR products

Products of PCR amplification of mtDNA regions were extracted from agarose gel and purified using a "DNA Cleanup" kit ("Bio-Rad", USA), as mentioned above. The nucleotide sequences of PCR-derived amplicons were done according to the method of Sanger (Sanger F). A Reagent kit ABI-PRISM(γ)BigDye™ Terminator V.3.1 (GE Healthcare, USA) was used, with a subsequent analysis of reaction products using an automated DNA sequencer ABI-PRISM 3730 (Applied Biosystems, USA). The sequences of interest were analyzed in both directions. The obtained reads of direct and reverse strands were compared pairwise to exclude the possible mistakes of sequencing.

Results

Experiments for identification of numt-pseudogenes originated *de novo* in the nuclear genomes of animal tissues require a thorough purification of analyzed nDNA samples free from impurities of mtDNA. For this reason, all samples of liver nDNA from each embryo were twice purified by gel-electrophoresis and tested for absence of free mtDNA impurities by the methods short and long extension PCR (Figure 1). Figure 1A represents three electrophoregrams of nuclear DNA isolated from chicken embryo liver. The first lane shows DNA obtained by a standard phenol-chloroform technique from lysed nuclei, without further electrophoretic purification, lanes 2 and 3 – samples resulting from electrophoretic separation of highest molecular fractions cut out from the gel for subsequent electrophoresis. It is clearly seen that the double electrophoresis of nDNA samples results in the removal of low-molecular impurities (Figure 1A, lane 3). The purified fractions of nDNA from each embryo were tested by PCR for the presence of impurities of free mtDNA, using primers flanking

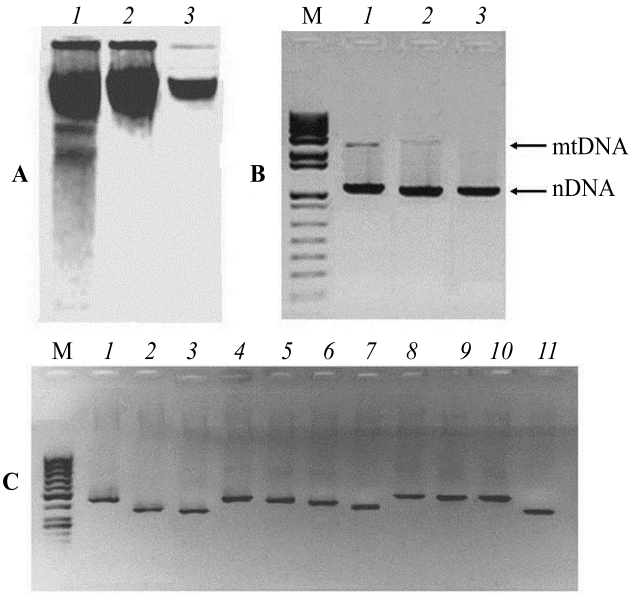

Figure 1: Purification of nDNA samples and testing the presence of impurities of free mtDNA by PCR. A–purification of nDNA by double gel-electrophoresis: 1, initial nDNA from nuclei of embryo liver; 2, after the first electrophoresis; 2, after the second electrophoresis. B- Gel-electrophoresis of PCR products of extensive mtDNA and nDNA regions (*β-actin* gene). Lane 1– initial nDNA from embryo liver nuclei, lanes 2 and 3– the same DNA samples after the first and the second rounds of purification by gel-electrophoresis. C: Gel-electrophoresis of PCR products obtained from nDNA isolated from liver nuclei (without electrophoretic purification) with 11 primer pairs listed in Table 1. M: DNA length markers.

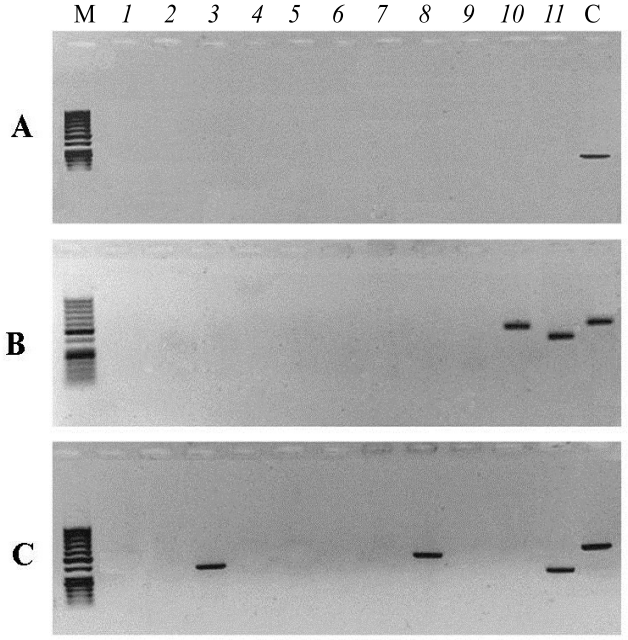

Figure 2: Gel-electrophoresis of PCR products from the templates of nDNA of embryos from unirradiated eggs (A) and embryos № 6 and № 7 from irradiated eggs (B, C), twice purified by electrophoresis. Lanes (1–11) – PCR with primer pairs listed in Table 1. C – control, PCR product from unpurified nDNA and primer pair 5 (Table 1). M: DNA length marker.

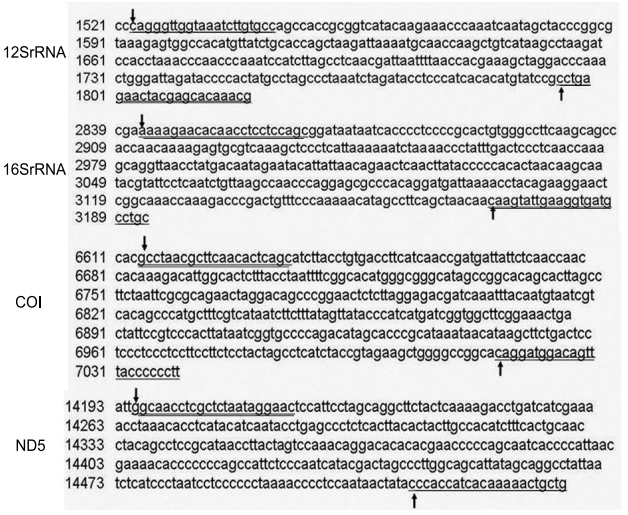

Figure 3: Nucleotide sequences (L-strands) of PCR amplicons synthesized from purified nDNA templates of embryo № 6 (12SrRNA, 16SrRNA) and № 7 (12SrRNA, COI, ND5) from irradiated eggs. Sequences which are underlined and marked with arrows correspond to direct and reverse primers (Table 1).

the extensive regions of mtDNA (15495 bp) and nDNA (*β-actin* 3193 bp) (Figure 1B). On the gel, it is seen that DNA samples isolated from nuclei (lane 1) contain impurities of mtDNA. However, it is obvious that using nDNA, twice purified electrophoretically, as a template for PCR gives no amplicons corresponding to mtDNA products (Figure 1B, lane 3). This means that double electrophoresis was successful for purification of nDNA samples from long fragments of free mtDNA. PCR analysis, involving nDNA as templates and 11 pairs of primers for mtDNA fragments, testified the presence of free mtDNA impurities in most nDNA samples of the first fraction (Figure 1C). Identical PCR tests (with 11 primer pairs) of electrophoretically purified samples of liver nDNA from chicken embryos taken out of unirradiated eggs gave negative results (Figure 2A). The obtained data prove that double electrophoresis allowed efficient purification of nDNA samples from free mtDNA fragments. Inserts of mtDNA were revealed in purified nDNA samples of embryos from 10 unirradiated eggs and of embryos from 8 irradiated eggs. PCR were run with each nDNA sample and with all 11 primer pairs for 3–4 times. Testing of 10 nDNA templates from control embryos with the abovementioned 11 primer pairs revealed no mtDNA sequences in them (Figure 2A exemplifies the results of such PCR analysis for one nDNA sample). At the same time, PCR with 11 primer pairs using nDNA templates isolated from irradiated egg embryos revealed amplified regions in two nDNA samples only. Stable detection was observed for PCR products from two and three loci of mtDNA in the nDNA samples of embryos № 6 and № 7, respectively.

Figure 2 shows electrophoregrams of PCR products synthesized from purified nDNA of embryos taken out of irradiated eggs (B – embryo № 6, C – embryo № 7). Numbers on the top part of electrophoregrams correspond to the primer pairs listed in Table 1. Lane C (control) corresponds to the PCR product obtained from electrophoretically unpurified nDNA of an unirradiated egg using primer pair 5 (Table 1). Figure 2 shows that the amplification of two mtDNA regions initiated by 10 and 11 pairs of primers occurs on the matrix of nDNA of embryo number 6. The amplification of three mtDNA regions initiated by 3, 8 and 11pairs of primers occurs on the matrix of the nDNA of embryo number 7. No synthesis was observed on the other six embryo nDNA samples from irradiated eggs (data not shown). These results indicate that the integration of mtDNA fragments into nDNA of liver took place in only two of the eight survived embryos from irradiated eggs. PCR- amplicons synthesized on nDNA templates of embryos from irradiated eggs (№ 6 and № 7) were excised from agarose gel, purified and sequenced as described earlier. The results of analysis are presented in Fig 3, where only the sequences of the L-strand of mtDNA are shown. Analyses show that the nucleotide sequences of amplicons are identical to mtDNA and to the sites of genes indicated (Figure 3). The sites of 12S rRNA and 16S rRNA genes are localized in the nDNA from embryo № 6, and the nDNA from embryo № 7 contains the sequences of COI, ND5 and 12S rRNA genes of mtDNA (Figure 3). Nucleotide sequence analysis also showed that the observed numt-insertions in nDNA contain rare single nucleotide polymorphisms that are not present in the homologous sites of free mtDNA. These polymorphisms are due to two or three substitutions of individual bases or deletions in the sequences of numt-inserts.

Thus, the results of this experiment confirm the assumption of the possibility of induction of numt-insertions into the nuclear genome of cells exposed to ionizing radiation. However, it can be assumed that the insertion of large fragments of mtDNA (300-400 bp) in nuclear genome, as in this experiment, is a rare event.

Discussion

Detection of large mtDNA sequences in the nuclear genome of chicken embryos developed from X-ray irradiated eggs indicates that in cells damaged by radiation, conditions are created for the formation of *de novo* numt-pseudogenes in nDNA. However, the insertions of mtDNA into liver nDNA were registered not in all embryos developed from irradiated eggs. Probably it depended on the conditions of the experiment, - first of all, on a limited number of chosen mtDNA sites and their large size (300-400 bp) for PCR amplification. Perhaps the amplification of small sizes mtDNA we could reveal more numt-inserts in the nDNA. In any case, the insertion of mtDNA fragments into nDNA sequences is presumably an infrequent event. It is also possible that the integration of mtDNA into the gene-coding nDNA sequences that are determinative of the development and survival of the embryo is lethal.

Analysis of literature data suggests that the frequency of "capturing" mtDNA by the nuclear genome depends on the rate of DSB occurring in nDNA, the activity of NHEJ repair systems, as well as on the amount of mtDNA fragments migrating into the nucleus [2,5,17-21]. Such a situation, beneficial for integration of mtDNA fragments into the nuclear genome, is developed following the exposure of the organism to some damaging agents, predominantly ionizing radiation [13,21]. MtDNA is currently considered to be a more sensitive target for different damaging factors compared to nDNA. For example, it has been previously demonstrated, that mtDNA gets more damage from ionizing radiation than a comparable fragment of nDNA in animal cells [13,27–29]. Moreover, mtDNA fragments are detected in the cytosol of brain and spleen cells of γ-irradiated mice for a long period of time [29,30]. Fragments of mtDNA might get into the intercellular space and the blood flow after radiation damage of the organism [30-32]. The release of mtDNA from mitochondria can occur as the result of their damage and selective mitophagy [13-15]. This phenomenon can also be favored by postradiational activation of ROS production in the mitochondria of damaged cells. It is well known, that the increased level of ROS can persist in mitochondria for a period from several minutes to several days, depending on the type of cells and radiation dose [33,34]. Therefore, it is safe to assume that mtDNA released from mitochondria gets into the cytosol; these molecules are likely to be protected from nuclease degradation by mitochondrial nucleoid proteins [35] and can migrate to the nuclei or between different cells [36].

It is a well-known fact that, along with various types of damage, ionizing radiation induces DSB in DNA. A predominant way of repair of DSB in nDNA in the cells of higher eukaryotes is the NHEJ mechanism, without the involvement of extensive regions on the ends of breaks [37]. DSB repair in nDNA can occur by the NHEJ mechanism, with the "capture" of an mtDNA fragment into end break joining sites, as well as with the involvement of microhomologies located in terminal sequences [38]. It has been recently demonstrated that the integration of mtDNA into nDNA can predominantly take place in the accessible regions of chromatin [39,40]. It is possible that new numt-pseudogenes also arise due to damage of DNA by other exogenous and endogenous factors. The integration of new mtDNA fragments apparently not only changes the structure of the genome in the regions of integration, but also substantially influences the realization of genetic information and genome stability. It has been demonstrated that the heat stress causes the migration of organelles mtDNA in the nucleus and their integration into the nuclear genome in plants [41]. A number of publications indicate that the amount of numt-insertions in nDNA grows with organism age. This may be due to accumulation of nDNA damage and mtDNA destruction due to action of ROS which are formed in the process of cell metabolism [12,35]. Recently it has been shown that nDNA in the tissues of old rats has a several-times higher homology with mtDNA, as compared to that of young animals [42]. Later on, the group of Barja G [12] used PCR and hybridization to prove that the number of mtDNA insertions raises in nDNA of liver and in the brain of rats with aging. The authors assume that accumulation of mtDNA insertions in animal nDNA might have a significant impact on organism aging [12]. This observation is confirmed by studies conducted on yeast cells, which testified the increase in the amount of numt-pseudogenes in nDNA with chronological aging [43].

As far back as 30 years ago it was proposed that the integration of mtDNA into the nuclear genome might be essential not only for aging, but also for cancer etiology [44]. For instance, the nDNA of rat hepatoma induced by a chemical carcinogen was found to contain homology regions with mtDNA (genes ND6 and COX1), which are not revealed in the nDNA of normal hepatocytes [45]. It was found that the mtDNA sequences (gene *COXIII*) are integrated into one of the two alleles of the nuclear gene *c-myc* in HeLa cells [46]. The nDNA of HeLa cells also contains nucleotide sequences of about 5 kb, corresponding to mtDNA genes: *COX1, ND4, ND4L* and *12S rRNA* [7]. The results of another study showed that the nDNA of tumor cells of mice and rats has more elements with mtDNA homology than the nDNA of their normal tissues [47]. The investigations of Liang [48] registered large fragments of mtDNA in the nDNA of human glioma, which are absent in the nDNA of neuroglial cells. According to another set of data, some mtDNA fragments are absent in the nDNA of normal cells but are integrated into the nDNA of cells of epithelial cervical cancer. There insertions of mtDNA strongly influence the expression of oncogene *c-myc* [11].

Additionally, there is evidence that there is a relation between some inherited diseases in humans and the appearance of mtDNA insertions in the encoded nuclear genes. It has been determined that six syndromes in humans are associated with the insertion of numt-pseudogenes in particular chromosomes [8-10,49-53]. Insertions of mtDNA in the coding gene of germ cells can be regarded as mutations, which can have serious consequences for embryogenesis and for the surviving of the offspring. It should be noted that the Pallister–Hall syndrome manifested as a functional disturbance of a key developmental gene as a result of a *de novo* insertion of an mtDNA fragment into the nuclear genome have been described earlier [8]. This mutation has appeared after insertion of an mtDNA fragment of 72 bp into the nucleotide sequence of the GLI3 gene in the 7th chromosome. The parents of the patient did not have such a mutation, and this mtDNA insertion could occur in the parental germ cell or at an early postzygotic stage of development of the baby. The family lived in a city with a high radiation level after the Chernobyl power plant accident [8].

Thus, the obtained experimental data suggest that ionizing radiation, which causes the destruction of mtDNA and DSB in nDNA and the activation of the repair system, can be regarded as a highly important factor capable of inducing *de novo* the generation of numt-pseudogenes.

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Authors Contributions

S.A.A. and A.I.G. were involved in project conception and design. S.A.A. was involved in laboratory processing and data acquisition for DNA isolation, PCR Array and DNA-sequences. A.I.G. helped interpret data and drafted the final manuscript, which was revised and approved by authors.

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