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MITOCHONDRIAL DNA AS AN OBJECT FOR BIODOSIMETRY AND BIOINDICATION OF ADDITIONAL IRRADIATION.

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If was shown that mtDNA «common deletion» can be used as a marker of irradiation for biological dosimetry.

Key words: biodosimetry, mitochondria, mtDNA.

МИТОХОНДРНАЛЬНАЯ ДНК КАК ОБЪЕКТ ДЛЯ БИОДОЗИМЕТРИИ И БИОИНДИКАЦИИ ПРИ ДОПОЛНИТЕЛЬНЫХ РАДИАЦИОННЫХ ВОЗДЕЙСТВИЯХ

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Показано, что «обычная делеция» мтДНК может быть использована как маркер облучения для биологической дозиметрии.

Ключевые слова: биодозиметрия, митохондрия, мтДНК.

In the course of investigation of the genetic processes after the irradiation the main attention has been concentrated on the state of nuclear DNA. At the same time the fact of the existence of the second alternative genome (in mammalian cells — mitochondrial DNA) is the well-known fact.

It is of a common knowledge, that in vertebrate cells mitochondrial DNA (mtDNA) is presented in multiply copies (10^3—10^4 per individual organism). Mammalian mitochondrial genome has the size in 16—18 kbp and ringed closed structure. Intermediate products analyses of mtDNA synthesis showed the main frames of the replication cycle [3, 4].

The universal nature of mtDNA and special lows of mitochondrial genetics (more related with population genetics lows that the lows of Mendel genetics) help to understand the very high genetic heterodiversity of mitochondrial diseases, which basing on the main involvement of the brain and muscular tissues got the common name «mitochondrial encephalomiopathy» [5, 9].

It was established that in human mtDNA there are so cold «hot sports» where mutations took place more often. The most important paradox of mitochondrial medicine [12] consists in inverted proportionality between number of the proteins, coded to two (nuclear and mitochondrial) genomes, and number of the diseases connected with their mutations. For the last few years in small mitochondrial genome that codes only 13 of the known approximately 1000 mitochondrial proteins (all 13 are the components of a respiratory chain), it was identified about 100 pathogenic dot mutations and reorganizations [13]. And, on the contrary, the numerous nuclear genome mutations could be associated with the respiratory chain defects and neural degenerative diseases rather rare.

The accumulated information confirms the involvement of mtDNA in the wide spectrum of pathologies [6]. There is suspicion that the level of mitochondrial mutations is the one of the cancer risk factors.

With the aging the frequency of mtDNA mutations is increasing [10] — first of all it concerns most frequently meeting «common deletion», covering 4977 base pairs. Its frequency grows up in the cells which have undergone a ultra-violet irradiation. There is opinion, that common deletion may be used

as a marker of a mutagenesis level of mitochondrial DNA.

The comparative analysis of effect of oxidizing stress on nuclear and mitochondrial DNA has shown that last one is more sensitive and the arisen anomalies are saved much longer in comparison with nuclear DNA [1]. The assumption was put forward, that its increased sensitivity to chemical and a physical mutagenic factors is connected with the absence histones [2]. This fact, and also absence in mitochondrion the sophisticated mechanism of a reparation [3], creates the real precondition to use the frequency of mtDNA aberrations for the purposes of biological dosimetry. Besides taking into account the fact of presence several mtDNA copies in every mitochondria it is possible to admit, that the aberration in one or several copies will not have an critical effect on organelle activity and cell — carrier viability as a whole and guarantees the co-existence of a plenty of mutations in physiologically high-grade cells.

The first attempts to use mtDNA («common deletion», 4977 bp) for the purposes of biodosimetry belongs to Kuboto et al [8], shown with the help nested PCR the increase of frequency of these deletions after irradiating.

We [11] using «Sunrise» type primers developed method for common deletion quantification by *in situ* PCR.

Materials and methods

Object of the investigations — human peripheral blood lymphocytes freshly purified in a density gradient with the standard technique.

Cell suspension subjected an *in vitro* irradiation in different dozes up to 2 Gy by ^{137}Cs source at the radiation power in 1 Gy/min.

Then cells were transferred into the standard lymphocyte cultural medium (RPMI-1640 medium, calf serum (15%), antibiotics), supplied by (according to experiment conditions) PHA (Gibco, 15 mkg/ml).

Then cells were washed in warm cultural medium without PHA(at +37°C, centrifugation — 5 min, 800g) and incubated in solution of Mitotraker M-7514 (0,2 mM in cultural medium, «Molecular probes») within 30 minutes. Then cells were washed in PBS, subjected hypotonic processing (PBS: distilled water = 1:1; +37°C, 8 minutes) also fixed 3 times in the cooled fixator (methanol: acetic acid = 3:1, 10 min). Intermediated centrifugation — 5 min, 800g.

Cellular mitochondrion identification (peripheral blood lymphocytes) with the help of mitotraker M-7514 (nuclear staining — DAPI).

Cells were dropped out on slides, dried up and used for PRINS PCR [7]. In experiments were used «Sunrise» type primers (HSAS8542) for identification mtDNA common deletions and standard kits «TaqMan PCR Core Reagent Core» (Perkin Elmer, H1555). Due to close positions of fluorochrom and quencher in primer, in solution it does not active. At interaction with mtDNA primer is stretching out and the distance between them is increasing and fluorescence will arise. Individual mutant mtDNA copies may be identified in such way.

For visualization of nuclei it was used standard DNA-specific counterstaining with DAPI. During the microscopic analysis the quantity of the cells with/without mutations were taken into account and expressed in percents.

Results and discussion.

Method development was carried out on lymphocyte culture of the patient with piers syndrome (higher frequency of common deletion)

Optimization of PRINS PCR regime (20—25 cycles) has allowed to identify with a high degree of reliability mutations of mtDNA with the help of the specified methodical approach — practically all cells contained mitochondria with mutant DNA, and the color varied significantly depending upon the number of mutant mtDNA copies from yellow — orange, up to intensively red.

At the analysis of the peripheral blood lymphocytes, irradiated by ^{137}Cs source in various dozes (up to 2 Gy) we fixed elevated levels of mutations (tab. 1), statistically significantly distinguished from the control.

Table 1

Dynamics of the mtDNA common deletion frequency in peripheral blood lymphocytes after an irradiation in various dozes (time of incubation — 0 hour.)

Dose, Gy	Amount of investigations	Cells in analyzes	Frequency of mutant cells, %
0	3	487	1,23±0,50
0,5	3	500	1,40±0,53
1,0	3	477	1,89±0,62
2,0	3	501	2,60±0,71

The analysis of the collected data specifies presence of statistically reliable difference only between control samples and the samples irradiated in a doze 2 Gy (according with Mann-Whitney criterion — $P < 0,05$).

Change of the mutant cells frequency depends on a doze and may be described in frameworks of regression model — $\beta = 0,74021$, $P < 0,01$ (fig. 1).

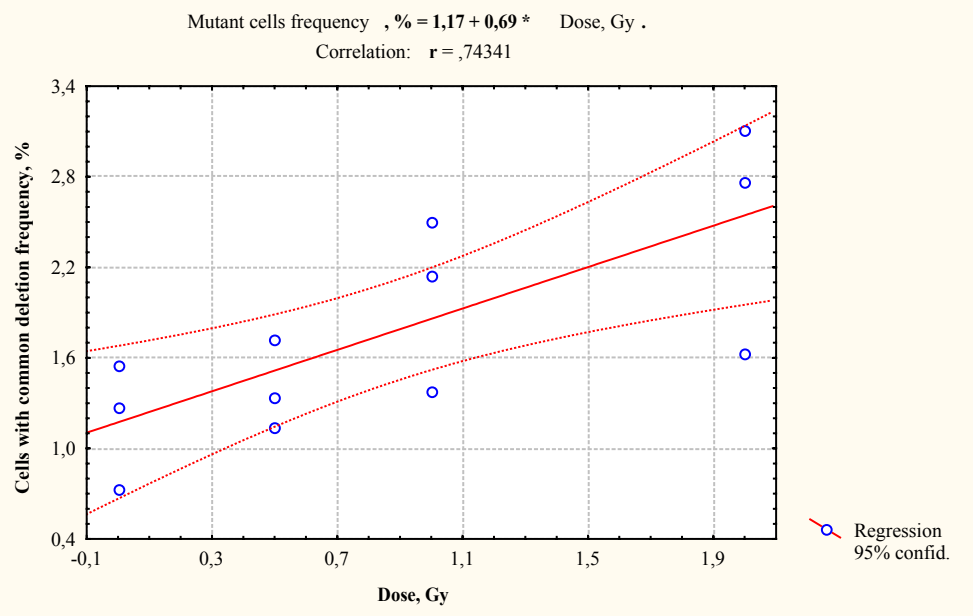

Fig. 1. Dynamics of the mtDNA common deletion induction after additional irradiation.

Unfortunately, the quantitative estimation of the mtDNA mutant frequency per one cell now is not possible, and in thanks to such situation we prefer to use as a basic criterion the frequency of mutant cells without dependence from the frequency of mutant organelles (or the damaged copies of a mitochondrial DNA).

Taking into account a possible(probable) role of the time factor and proliferation poten-

tial in realization of effects of radiating influence, we carry out (spend) the analysis of frequency of an induction of deletions of a mitochondrial DNA for the same dose range in 96 hour. (an estimation of a role of the time factor) after an irradiation without / at presence PHA (a role proliferation potential).

Results of researches are summarized in tab. 2 and 3.

Table 2

Dynamics of the mtDNA common deletion frequency in peripheral blood lymphocytes after an irradiation in various dozes (time of incubation - 96 hour, without PHA)

Dose, Gy	Amount of investigations	Cells in analyzes	Frequency of mutant cells, %
0	3	754	0,93±0,35
0,5	2	990	3,43±0,58
1,0	3	960	15,73±1,18
2,0	3	1082	16,73±1,14

Table 3

Dynamics of the mtDNA common deletion frequency in peripheral blood lymphocytes after an irradiation in various dozes (time of incubation - 96 hour, with PHA)

Dose, Gy	Amount of investigations	Cells in analyzes	Frequency of mutant cells, %
0	3	938	1,60±0,41
0,5	3	1158	13,04±0,99
1,0	3	1102	22,60±1,26
2,0	3	1205	27,14±1,28

The analysis of the data presented in Table 2 testifies that frequency of cells with deleted mitochondrial DNA is really increased in the dose range 0—1 Gy (Mann-Whitney criterion, for all cases — $P < 0,05$). But in a range of dozes 1—2 Gy the output of mutant cells exits on a plateau ($P > 0,05$).

At the same time, in case of stimulation cellular пролиферации frequency of mutant cells is much higher, and the difference between points 1 and 2 Gy is statistically reliably different (Mann-Whitney criterion, for all cases — $P < 0,05$).

It is clear that in both cases the doze — effect dependence may be interpreted in the framework of linear regression - in the case non-stimulated lymphocytes $\beta = 0,890$ ($P < 0,001$); after PHA stimulation — $\beta = 0,930$ ($P < 0,00005$) (fig. 2).

The special attention deserves the fact, that the frequency of mutant cells submits to the same law both in PHA-stimulated, and in PHA-unstipulated cell populations that enables to assume relative independence of the specified parameter from proliferation activity of lymphocytes, and the marked quantitative difference can be attributed to the account of duplication of cells — carriers of a mutated mtDNA.

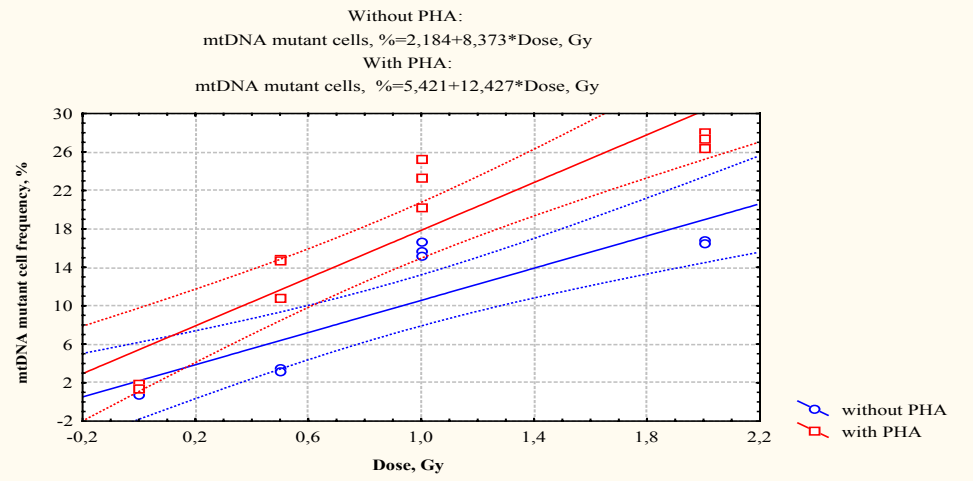

Fig. 2. Dynamics of mtDNA common deletion induction after additional irradiation (incubation time — 96 h) / without the PHA stimulation.

At the same time the comparison of the regression equations, devoted to the dose-effect dependencies in these cases testifies that between them there are real statistically reliable discrepancy ($P<0,01$).

The above mentioned data allow making the following conclusions:

- The analysis of the non-nuclear DNA condition is an informative parameter for biological dosimetry;
- Relative independence of the frequency of mutant cells from cell proliferation activity of the lymphocytes allows assuming the possibility long-time stability of this parameter.

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