PROCEEDINGS OF THE YEREVAN STATE UNIVERSITY

Chemistry and Biology 2020, **54**(1), p. 63–67

Biology

IDENTIFICATION OF CYTOGENETICALLY VISIBLE mtDNA IN INTERPHASE NUCLEI OF HUMAN BLOOD LYMPHOCYTES EXPOSED TO DOXORUBICIN

T. A. HARUTYUNYAN [1*], G. G. HOVHANNISYAN [1**], A. A. SARGSYAN [1***], T. LIEHR [2****], A. AL-RIKABI [2*****], R. M. AROUTIOUNIAN [1******]

[1] *Chair of Genetics and Cytology, YSU, Armenia*
[2] *Laboratory of Molecular Cytogenetics, Institute of Human Genetics Jena, Germany*

The endosymbiotic hypothesis suggests that mitochondria were free-living organisms that colonized another cells, and millions of years of co-evolution resulted in the transfer of a large number of genes from mitochondria to the nucleus. Recent studies indicate that this is an ongoing process and can be due to the impact of cells to DNA damaging agents, e.g. radiation or chemical mutagens. Here we demonstrate that cytogenetically visible mitochondrial DNA translocation into interphase nuclei genome in human blood lymphocytes can be identified via fluorescence in situ hybridization after exposure of cells to doxorubicin.

***Keywords*:** mtDNA, fluorescence in situ hybridization, human blood, doxorubicin.

Introduction. Mitochondria are known as the “powerhouse” of eukaryotic cells (i.e. ATP synthesis organelle) which carries up to several copies of circular DNA each containing 16.569 bp and 37 genes, and the number of mitochondria per cell can range from less than one hundred to several thousand [1]. Thus, in addition to the nuclear genome, each human cell carries 1–50 *Mb* of mitochondrial DNA (mtDNA) taken together. The endosymbiotic hypothesis suggests that mitochondria were free-living organisms that colonized another cell, and millions of years of co-evolution resulted in the transfer of a large number of genes from mitochondria to the nucleus [2]. There are two types of mtDNA in the nucleus. The first are nuclear DNA sequences encoding mitochondrial gene products that are no longer present in mitochondria. The second are nuclear DNA sequences that are copies of existing mtDNA. These sequences, called nuclear DNA sequences of mitochondrial origin or NUMTs, can be detected in a variety of eukaryotic nuclear genomes [3].

During the last decade whole-cancer genomes and their counterparts in normal tissues have been sequenced and compared, which allowed revealing

[*] E-mail: tigranharutyunyan@ysu.am
[**] E-mail: hovgalina@list.ru
[***] E-mail: angela.sargsyan@mail.ru
[****] E-mail: thomas.liehr@med.uni-jena.de
[*****] E-mail: ahmed.al-rikabi@med.uni-jena.de
[******] E-mail: rouben_a@hotmail.com

structural variations in the genome-specific to cancer cells. Recently, somatic nuclear mtDNA transfers (SNMTs) into the nuclear genome have been observed from breast cancers and several cancer cell lines. Analysis of 587 pairs of cancer and normal whole-genome sequencing data revealed 25 mitochondrial-nuclear DNA integration events and a somatically acquired integration of almost the entire mtDNA sequence (16.556 bp) into chromosome 10q22.3 in breast cancer cells [4]. Nevertheless, the frequency of SNMTs across different cancer tissue types is variable. It was shown, that triple-negative breast cancers have a 5-fold higher rate of SNMTs compared to estrogen-receptor-positive breast cancers (6.2% vs. 1.2%, respectively) [4]. At the same time, colorectal tumors detected in women contained more NUMTs than in men (4.52 fold vs 3.51 fold, respectively) indicating sex-differences in the SNMT rate [5]. Although the cell transformation stage, at which SNMTs frequently occur, remains to be determined.

The results of several studies suggest that NUMTs are distributed non-randomly and tend to be located in damage-prone regions of the nuclear genome such as open chromatin and fragile sites [6]. In studies with yeast it was demonstrated that incorporation of fragmented mtDNA into the nuclear genome occurs during the repair of induced DNA double-stranded breaks (DSBs) of nuclear DNA [7]. Thus, we hypothesized that agents capable to induce DSBs, including chemical and physical mutagens, have the potential to influence the migration of mtDNA into the nuclear genome. To investigate this hypothesis, we analyzed the frequency of cytogenetically visible mtDNA translocation into the nuclear genome after exposure of human whole blood culture to genotoxic anticancer drug doxorubicin (DOX) using fluorescence in situ hybridization (FISH).

Materials and Methods.

Cell Cultures. Blood samples were collected by venipuncture from six healthy nonsmoking donors (three female and three male) aged 27–29 years. This study was approved by the Ethics Committee of the National Center of Bioethics (Yerevan State University, Faculty of Biology), and informed consent was obtained from all study donors. The venous blood (2 *mL* from each donor) was collected into vacutainers with heparin. Blood samples were cultivated in RPMI-1640 medium, containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 $\mu g/mL$ phytohemagglutinin-L at 37°*C* for 72 *h*.

DOX Treatment of Human Blood. For induction of mtDNA transfer into nuclear genome human whole blood was treated with 0.0175 and 0.035 $\mu g/mL$ of DOX for 24 *h* at 37°*C*. The implemented doses were selected based on [8], which investigated the DNA damaging action of DOX. 500 interphase nuclei were analyzed in each experimental variant.

FISH Probe Generation for mtDNA. FISH probe for mtDNA (mtFISH) was prepared using 9 pairs of primers designed for amplification of the whole mitochondrial genome (Tab. 1). Each reaction mixture contained 2 μL of a buffer, 2 μL of Label-mix, 2 μL of $MgCl_2$ (25 *mM*), 0.12 μL of AmpliTaq DNA Polymerase, 2 μL of fluorochromes and 12.08 μL of H_2O.

***mtFISH Analysis*.** FISH analysis was previously used for the detection of mtDNA insertion in interphase nuclei [4]. Slides were dehydrated with ethanol series (70, 95 and 100%), treated with pepsin for 5 *min* at 37°*C* in the water bath, washed in PBS for 5 *min* at room temperature, incubated with 100 μL of Postfixation solution (5 *mL* paraformaldehyde + 4.5 *mL* PBS + 500 μL 1 *M* $MgCl_2$)

for 10 *min* at room temperature under a flow hood. After final dehydration series with ethanol (70, 95, and 100%) the slides and probes were denatured at 81°*C* and hybridized overnight in a humid chamber at 37°*C*. The slides were washed with SSC solution and analyzed under a Zeiss fluorescent microscope using 100×objective. 500 interphase nuclei were analyzed in each treatment and control variant. Statistical analysis was performed using Student's *t*-test.

Table 1

Primer set for whole mitochondrial genome amplification.
PCR product size and sequences of forward (for) and reverse (rev) primers are presented [9]

Product length, bp	Sequence (5'–3')
1822	for – tagccatgcactactcaccaga rev – ggatgaggcaggaatcaaagac
1758	for – ctgtatccgacatctggttcct rev – gtttagctcagagcggtcaagt
2543	for – acttaagggtcgaaggtggatt rev – tcgatgttgaagcctgagacta
3005	for – aagtcaccctagccatcattcta rev – gatatcatagctcagaccatacc
2709	for – ctgctggcatcactatactacta rev – gattggtgggtcattatgtgttg
1738	for – cttaccacaaggcacacctaca rev – ggcacaatattggctaagaggg
1866	for – gtctggcctatgagtgactaca rev – cagttcttgtgagctttctcgg
1853	for – ctccctctacatatttaccacaac rev – aagtcctaggaaagtgacagcga
1872	for – gcaggaatacctttcctcacag rev – gtgcaagaataggaggtggagt

Results and Discussion. We have studied the frequency of translocation of mtDNA into the nuclear genome in human interphase nuclei after exposure of whole blood cultures to DOX (0.0175 $\mu g/mL$ and 0.035 $\mu g/mL$) for 24 *h*.

Table 2

*Number of mtFISH-positive nuclei per 500 cells after treatment of human whole blood cultures with DOX (*p<0.05 – significant difference compared to control)*

Donors	Control (mean ± SD)	DOX 0.0175 $\mu g/mL$ (mean ± SD)	DOX 0.035 $\mu g/mL$ (mean ± SD)
D1	7.5 ± 2.1	9.5 ± 2.1	16.0 ± 1.4*
D2	11.0 ± 1.4	11.5 ± 0.7	16.5 ± 0.7
D3	5.5 ± 0.7	7.5 ± 0.7	9.0 ± 1.4
D4	9.0 ± 2.8	11.0 ± 1.4	16.0 ± 2.8*
D5	5.5 ± 0.7	4.5 ± 0.7	11.0 ± 0.7*
D6	3.5 ± 0.7	6.5 ± 0.7	6.5 ± 0.7*

The obtained results revealed a significant elevation of the frequency of mtFISH-positive nuclei after treatment with DOX (Tab. 2). In the control group the frequency of mtFISH-positive nuclei ranged from 3.5 ± 0.7 to 11 ± 1.4. In the groups treated with 0.0175 $\mu g/mL$ and 0.035 $\mu g/mL$ DOX the frequencies of

mtFISH-positive nuclei ranged from 4.5±0.7 to 11.5±0.7 and 6.5±0.7 to 16.5±0.7 ($p<0.05$), respectively.

Significant positive correlations between number of mtFISH-positive nuclei and 0.0175 *μg/mL* (r=0.868; p=0.0003) and 0.035 *μg/mL* (r=0.834; p=0.0007) DOX treatment concentrations was, which indicates dose-dependent effect of mtDNA transfer into nucleus.

It was shown that the human reference genome (version hg19) contains 755 NUMTs, and the largest human NUMT covers 90% (14.654 bp) of the human mitochondrial genome [10]. In our study mtFISH-positive nuclei were identified in the control group with different frequencies ranging from 3.5 ± 0.7 to 11.0 ± 1.4 nuclei per 500 nuclei. These results indicate the interindividual difference in the distribution of spontaneous NUMTs in cells of whole blood.

Based on studies in yeast it was suggested that mtDNA insertions were mediated by nuclear DNA DSB repair via activation of non-homologous end joining, microhomology-mediated end-joining or homologous recombination pathways [7].

In our study treatment of whole blood culture with DOX resulted in an elevation of the number of nuclei with mtFISH signals. Interestingly, it was also demonstrated that the treatment of hepatocytes with DOX significantly increases non-homologous end-joining activity in vitro [11]. Thus, our results provide additional data indicating potential involvement of DNA damage and cellular response as key players in mtDNA translocation into the nuclear genome.

In conclusion, the obtained results confirm previous studies indicating that DNA DSB-inducing mutagens have the potential to induce translocation of mtDNA fragments into the nuclear genome. The mt-FISH approach is suitable for the detection of large mtDNA insertions, while small fragments, which probably may prevail, will be underestimated.

This work was supported by the SC MES RA-BMBF (AG-01/18).

Received 24.02.2020
Reviewed 24.03.2020
Accepted 10.04.2020

REFERENCES

1. Ju Y.S. Intracellular Mitochondrial DNA Transfers to the Nucleus in Human Cancer Cells. *Curr Opin Genet Dev.*, **38** (2016), 23–30.
2. Leister D. Origin, Evolution and Genetic Effects of Nuclear Insertions of Organelle DNA. *Trends Genet.*, **21** (2005), 655–663.
3. Hazkani-Covo E., Zeller R.M., Martin W. Molecular Poltergeists: Mitochondrial DNA Copies (numts) in Sequenced Nuclear Genomes. *PLoS Genet.*, **6** (2010), e1000834.
4. Ju Y.S., Tubio J.M., Mifsud W., Fu B., Davies H.R., Ramakrishna M., Li Y., Yates L., Gundem G., Tarpey P.S. et al. Frequent Somatic Transfer of Mitochondrial DNA into the Nuclear Genome of Human Cancer Cells. *Genome Res.*, **25** : 6 (2015), 814–824.
5. Srinivasainagendra V., Sandel M.W., Singh B., Sundaresan A., Mooga V.P., Bajpai P., Tiwari H.K., Singh K.K. Migration of Mitochondrial DNA in the Nuclear Genome of Colorectal Adenocarcinoma. *Genome Med.*, **9** : 1 (2017), 31 p.
6. Wang D., Timmis J.N. Cytoplasmic Organelle DNA Preferentially Inserts into Open Chromatin. *Genome BiolEvol.*, **5** (2013), 1060–1064.
7. Decottignies A. Capture of Extranuclear DNA at Fission Yeast Double-Strand Breaks. *Genetics*, **171** : 4 (2005), 1535–1548.

8. Yang F., Kemp C.J., Henikoff S. Anthracyclines Induce Double-Strand DNA Breaks at Active Gene Promoters. *Mutat Res*., **773** (2015), 9–15.
9. Ramos A., Santos C., Alvarez L., Nogués R., Aluja M.P. Human Mitochondrial DNA Complete Amplification and Sequencing: a New Validated Primer Set that Prevents Nuclear DNA Sequences of Mitochondrial Origin co-Smplification. *Electrophoresis*, **30** : 9 (2009), 1587–1593.
10. Mourier T., Hansen A.J., Willerslev E., Arctander P. The Human Genome Project Reveals a Continuous Transfer of Large Mitochondrial Fragments to the Nucleus. *Mol. Biol. Evol*., **18** : 9 (2001), 1833–1837.
11. Yang S., Wang X.Q. XLF-mediated NHEJ Activity in Hpatocellular Carcinoma Therapy Resistance. *BMC Cancer*, **17** : 1 (2017), 344 p.

Տ. Ա. ՀԱՐՈՒԹՅՈՒՆՅԱՆ, Գ. Գ. ՀՈՎՀԱՆՆԻՍՅԱՆ, Ա. Ա. ՍԱՐԳՍՅԱՆ,
Թ. ԼԻՐ, Ա. ԱԼ-ՌԻԿԱԲԻ, Ռ. Մ. ՀԱՐՈՒԹՅՈՒՆՅԱՆ

ԲՋՋԱԳԵՆԵՏԻԿՈՐԵՆ ՏԵՍԱՆԵԼԻ մտԴՆԹ-Ի ՆՈՒՅՆԱԿԱՆԱՑՈՒՄԸ ԴՈՔՍՈՌՈՒԲԻՑԻՆՈՎ ՄՇԱԿՎԱԾ ՄԱՐԴՈՒ ԱՐՅԱՆ ԼԻՄՖՈՑԻՏՆԵՐԻ ԻՆՏԵՐՖԱԶԱՅԻՆ ԿՈՐԻԶՆԵՐՈՒՄ

Ամփոփում

Էնդոսիմբիոզի տեսությանը համաձայն միտոքոնդրիումները եղել են ազատ ապրող օրգանիզմներ, որոնք սիմբիոզի մեջ են մտել մեկ այլ բջջի հետ և միլիոնավոր տարիների համաէվոլյուցիան հանգեցրել է միտոքոնդրիումից դեպի բջջի կորիզային գենոմ բազմաթիվ գեների տեղափոխման: Վերջին տարիների աշխատանքները ցույց են տալիս, որ գեների տեղափոխման այս երևույթը հանդիպում է ԴՆԹ-ի վնասվածքներ առաջացնող գործոնների (օրինակ՝ քիմիական և ֆիզիկական մուտագեններ) ազդեցությամբ: Ստացված արդյունքները ցույց տվեցին, որ դոքսոռուբիցինով մարդու արյան լիմֆոցիտների մշակումը հանգեցնում է կորիզային գենոմում բջջագենետիկորեն տեսանելի մտԴՆԹ-ի հանդիպման հաճախականության բարձրացման:

Т. А. АРУТЮНЯН, Г. Г. ОГАНЕСЯН, А. А. САРГСЯН, Т. ЛИР,
А. АЛ-РИКАБИ, Р. М. АРУТЮНЯН

ИДЕНТИФИКАЦИЯ ЦИТОГЕНЕТИЧЕСКИ ВИДИМОЙ МИТОХОНДРИАЛЬНОЙ ДНК В ИНТЕРФАЗНЫХ ЯДРАХ ЛИМФОЦИТОВ КРОВИ ЧЕЛОВЕКА ПРИ ВОЗДЕЙСТВИИ ДОКСОРУБИЦИНА

Резюме

Эндосимбиотическая гипотеза предполагает, что митохондрии были свободными организмами, которые колонизировали другие клетки, и миллионы лет коэволюции привели к переносу большого количества генов из митохондрий в ядерный геном. Недавние исследования показали, что перемещение митохондриальных генов в ядро может происходить при действии на клетки генотоксических факторов (химических и физических мутагенов). Полученные результаты выявили, что обработка клеток крови человека доксорубицином повышает частоту интерфазных ядер с цитогенетически видимой митохондриальной ДНК.