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Mitochondrial Genome Sequencing in Atherosclerosis: What's Next?

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Abstract: Cardiovascular diseases are currently a basic cause of mortality in highly developed countries. The major reason for genesis and development of cardiovascular diseases is atherosclerosis. At the present time high technology methods of molecular genetic diagnostics can significantly simplify early presymptomatic recognition of patients with atherosclerosis, to detect risk groups and to perform a family analysis of this pathology.

A Next-Generation Sequencing (NGS) technology can be characterized by high productivity and cheapness of full genome analysis of each DNA sample. We suppose that in the nearest future NGS methods will be widely used for scientific and diagnostic purposes, including personalized medicine.

In the present review article literature data on using NGS technology were described in studying mitochondrial genome mutations associated with atherosclerosis and its risk factors, such as mitochondrial diabetes, mitochondrial cardiomyopathy, diabetic nephropathy and left ventricular hypertrophy.

With the use of the NGS technology it proved to be possible to detect a range of homoplasmic and heteroplasmic mutations and mitochondrial genome haplogroups which are associated with these pathologies. Meanwhile some mutations and haplogroups were detected both in atherosclerosis and in its risk factors. It conveys the suggestion that there are common pathogenetic mechanisms causing these pathologies.

What comes next? New paradigm of crosstalk between non-pharmaceutical (including molecular genetic) and true pharmaceutical approaches may be developed to fill the niche of effective and pathogenically targeted pretreatment and treatment of preclinical and subclinical atherosclerosis to avoid the development of chronic life-threatening disease.

Keywords: Next-Generation Sequencing, whole mitochondrial genome, gene, mutation, heteroplasmic, homoplasmic, atherosclerosis, risk factors of atherosclerosis.

INTRODUCTION

Cardiovascular diseases are currently a basic cause of mortality in highly developed countries [1-10]. The major reason for genesis and development of cardiovascular diseases is atherosclerosis [1, 11-18]. At the present time high technology methods of molecular genetic diagnostics can significantly simplify early presymptomatic recognition of patients with atherosclerosis, to detect risk groups and to perform a family analysis of this pathology [19-27].

The most promising method for detection of mutations associated with different pathologies is sequencing of a patient`s DNA. More than twenty years ago automated Sanger sequencing was used as a basic method for direct analysis of DNA nucleotide sequence. A disadvantage of this method is a limited length of a DNA fragment which can be read, approximately 1000 nucleotides. This method belongs to "the first generation sequencing". A Next-Generation Sequencing (NGS) technology is a later development. NGS methods can be characterized by high productivity and cheapness of full genome analysis of each DNA sample. A cost of sequencing declines steadily every next year. We suppose that in the nearest future NGS methods will be widely used for scientific and diagnostic purposes, including personalized medicine [28, 29].

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The mainest difference of Next-Generation Sequencing from Sanger sequencing (capillary sequencing) is a multiple, simultaneous reading of short DNA fragments. Meanwhile nucleotide sequences of these fragments overlay. It guarantees the absence of unread genome segments. Several types of equipment for NGS were developed, but most of these technologies have a common algorythm of nucleotide sequence detection. Firstly, libraries of an analyzed DNA sample are created, which are basically DNA fragments with overlapping sequences. At the next stage a clonal amplification of these fragments takes place. It is necessary for obtaining a "matrix" for sequencing. After that a direct reading of a nucleotide sequence occurs [30].

The most important advantages of NGS compared to other methods of molecular genetic analysis are:

- A high productivity, because of which it is possible to detect mutations and polymorphisms of the whole genome by one analysis of a DNA sample [31].
- A high probability of new mutations and polymorphisms detection. Previous methods, used in molecular genetic researches, such as Sanger sequencing, PCR, miroarray analysis detected a limited number of mutations and polymorphisms. As a result, rare variants which were not in a set of chosen molecular genetic markers for the analysis remained not detected [29].
- A high sensibility. Due to the NGS method, a possibility of detection of mutations in a small amount of analyzed biomaterial cells has appeared [32].

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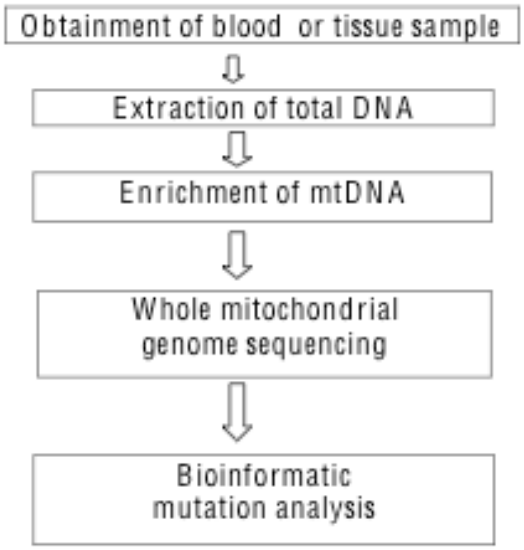

Fig. (1). Algorithm whole mitochondrial genome mutations analysis in atherosclerosis using next-generation sequencing.

Methods used for the whole mitochondrial genome analysis by the NGS technology

Too little of mitochondrial DNA is extracted from patient's blood cell and tissue samples for molecular genetic investigations. This is the reason why firstly total DNA is extracted. Then, using special primers in PCR, the number of mitochondrial genome copies is increased.

At present there are two such methods:

- An enrichment of mtDNA performed using Qiagen™ REPLI-g Mitochondrial Kit [33, 38].
- Amplification of mtDNA from genomic DNA using Roche Expand Long Range PCR dNTPack (Roche Applied Science, Indianapolis, IN) [37, 39, 40].

Then in the enriched mtDNA samples the whole spectrum of mutations is analyzed using Next-Generation sequencing technologies. With the use of bioinformatic programs mitochondrial genome mutations associated with the investigated pathologies are identified.

Sequeners used for whole mitochondrial genome analysis by the Next-Generation Sequencing technology

Due to the fact that for the investigation of mitochondrial genome heteroplasmy level it is necessary to conduct a quantitative analysis of mutations, for NGS, the sequencers of the following companies are most frequently used:

1) Roche;

2) Applied Biosystems.

For example, for NGS scientists use such sequencers as Roche 454 GS Junior Titanium system (Roche Diagnostics GmbH) and ABI-Prism 3730 Genetic Analyzer (Applied Biosystems).

Analysis of whole mitochondrial genome mutations in carotid atherosclerosis with the use of NGS

Nowadays it is reported that there appeared investigations in which an association of mitochondrial genome mutations with carotid atherosclerosis was found by the NGS method. Specifically, such a work was done by Russian scientists [33]. A whole mitochondrial genome analysis was performed for a sample of 60 individuals who were diagnosed with atherosclerosis on grounds of ultrasonograpfic investigations and biochemical tests.

To enrich mitochondrial DNA Qiagen™ REPLI-g Mitochondrial Kit was used. A mitochondrial genome analysis was conducted by Roche 454 GS Junior Titanium system (Roche Diagnostics GmbH).

In the investigated sample 422 homoplasmic mitochondrial genome variants were found. Among them 59 mitochondrial genome mutations were associated with atherosclerotic lesions of carotid arteries. Mitochondrial mutations m.5147G>A and m.930G>A were distinguished only in healthy individuals (Table 1). At the same time frequency of 24 mutations (m.146T>C, m.185G>A, m.195T>C, m.462C>T, m.489T>C, m.709G>A, m.1811A>G, m.1888G>A, m.4216T>C, m.8697G>A, m.11251A>G, m.11812A>G, m.12612A>G, m.13708G>A, m.14233A>G, m.14798T>C, m.14905G>A, m.15452C>A, m.15607A>G, m.15928G>A, m.16069C>T, m.16126T>C, m.16294C>T and m.16296C>T) in a control sample was from 2 to 5-fold higher than in a sample of individuals with atherosclerotic lesions of carotid arteries (Table **1**).

Mitochondrial genome mutations m.204T>C, m.3010G>A, m.8251G>A and m.12705C>T were found to be associated with atherosclerosis as their frequency in patients with atherosclerotic lesions was 1.6-fold higher than in healthy people (Table **1**).

Russian scientists also found 104 heteroplasmic mitochondrial mutations. 72 of them were localized in the coding regions. Meanwhile five mutations of mitochondrial genome coding region occurred significantly more often in a sample of healthy individuals, than in atherosclerotic patients (Table **2**). At the same time mutations m.9477G>A and m.8528insA were predominant in patients with atherosclerosis.

Researchers remarked that previously, using pyrosequenator PSQ 96MA system (Biotage, Sweden) eleven mitochondrial genome heteroplasmic mutations associated with atherosclerotic lesions have been detected [34-36]. Data on these mutations are represented in Table **3**.

These mutations were not found in this study [33]. However, in both studies an association of mutant variants of genes MT-ND1, MT-ND2, MT-ND5, MT-ND6 and MT-CYTB with atherosclerotic lesions was found [33-36].

The data, listed below, which compare two different methods: Sanger sequencing and NGS, prove that with the help of various sequencing methods it is possible to detect a different list of mutations [33-36, 38].

Investigation of mitochondrial mutations with the use of NGS in pathologies, which are risk factors of atherosclerosis

1. MITOCHONDRIAL GENOME MUTATIONS IN MATERNALLY INHERITED CARDIOMYOPATHY

1.1. A Homoplasmic Mitochondrial Mutation Associated with Maternally Inherited Hypertrophic Cardiomyopathy

Mutations in mitochondrial DNA can be a cause of maternally-inherited cardiomyopathy. In a collaborative study of Italian, British and American researchers a family analysis of mitochondrial genome mutations in two families with maternally inherited hypertrophic cardiomyopathy was performed [37]. The number of members in the first family was 4 and in the second family there were 24 members. Sequencing of the investigated mtDNA samples was performed using Applied Biosystems 377 automated DNA sequencer (Foster City, California).

In both families a homoplasmic mutation 4300A>G (gene MT-TI), associated with maternally inherited hypertrophic cardiomyopathy was detected.

Table 1. Common homoplasmic mitochondrial genome mutations associated with atherosclerosis.

Gene or region	Mutation	Atherogenic effect	Antiatherogenic effect
D-loop region	m.146T>C	-	+
	m.185G>A	-	+
	m.195T>C	-	+
	m.204T>C	+	-
	m.462C>T	-	+
	m.489T>C	-	+
Gene tRNA-Phe (MT-TF)	m.709G>A	-	+
	m.930G>A	-	+
Gene rRNA 16S (MT-RNR2)	m.1811A>G	-	+
	m.1888G>A	-	+
	m.3010G>A	+	-
Gene NADH dehydrogenase subunit 1 (MT-ND1)	m.4216T>C	-	+
Gene NADH dehydrogenase subunit 2 (MT-ND2)	m.5147G>A	-	+
Gene cytochrome C oxidase subunit II (MT-COX2)	*m.8251G>A*	+	-
Gene ATP synthase subunit 6 (MT-ATP6)	m.8697G>A	-	+
Gene NADH dehydrogenase subunit 4 (MT-ND4)	m.11251A>G	-	+
	m.11812A>G	-	+
Gene NADH dehydrogenase subunit 5 (MT-ND5)	m.12612A>G	-	+
	m.12705C>T	+	-
	m.13708G>A	-	+
Gene NADH dehydrogenase subunit 6 (MT-ND6)	m.14233A>G	-	+
Gene cytochrome B (MT-CYTB)	m.14798T>C	-	+
	m.15452C>A	-	+
	m.15607A>G	-	+
Gene tRNA-Thr (MT-TT)	m.15928G>A	-	+
D-loop region	m.16069C>T	-	+
	m.16126T>C	-	+
	m.16294C>T	-	+
	m.16296C>T	-	+

1.2. Several Mitochondrial Mutations Associated with Maternally Inherited Cardiomyopathy

In a collaborative study of scientists from the USA and Italy 18 patients with mitochondrial cardiomyopathy and two with suspected mitochondrial disease were investigated [38]. In this study scientists compared the sensitivity of two detection methods of mitochondrial genome mutations in patients with maternally inherited cardiomyopathy:

1) Next-generation sequencing;

2) Sanger sequencing.

An enrichment of mtDNA was performed using Roche Expand Long Range PCR dNTPack kit (Roche Applied Science, Indianapolis, IN). An analysis of whole mitochondrial genome was performed by Roche's 454 Genome Sequencer. Using each of these methods scientists managed to identify more than 400 mutations. Meanwhile method of NGS allowed detecting 98% of mutations, which were identified by Sanger method. Some of mutations, found with the use of Roche sequenator were not identified during Sanger analysis.

Table 2. Common heteroplasmic mitochondrial genome mutations associated with atherosclerosis.

Gene	Mutation	Atherogenic effect	Antiatherogenic effect
ATP synthase subunit 8 (MT-ATP8)	m.8516insA	-	+
	m.8516insC	-	+
	m.8528insA	+	-
ATP synthase subunit 6 (MT-ATP6)	m.8930insG	-	+
Cytochrome C oxidase subunit III (MT-COX3)	*m.9477G>A*	+	-
NADH dehydrogenase subunit 4 (MT-ND4)	m.10958insC	-	+
NADH dehydrogenase subunit 5 (MT-ND5)	m.13050insC	-	+

Table 3. Major heteroplasmic mitochondrial mutations in atherosclerosis, detected using PSQ 96MA system (Biotage, Sweden)

Gene	Mutation	Atherogenic effect	Antiatherogenic effect
12S rRNA (MT-RNR1)	*m.652delG*	+	-
	m.652insG	-	+
	m.A1555G	-	+
tRNA - Leu (codon recognized UUR) (MT-TL1)	*m.C3256T*	+	-
MT-ND1	*m.T3336C*	+	-
MT-ND2	*m.C5178A*	+	-
tRNA - Leu (codon recognized CUN) (MT-TL2)	*m.G12315A*	+	-
MT-ND5	m.G13513A	-	+
MT-ND6	*m.G14459A*	+	-
MT-CYTB	m.G14846A	-	+
	m.G15059A	+	-

Therefore with the use of Next-Generation Sequencing eight mutations associated with mitochondrial cardiomyopathy were detected (Table **4**). Four heteroplasmic mutations of them were distinguished by both methods (Sanger and NGS). Besides, two homoplasmic mitochondrial genome mutations detected by Sanger method were not found by Next-Generation Sequencing.

It is notable that homoplasmic mutation mt3010G>A was associated both with atherosclerotic lesion of vessels and atherosclerotic risk factor, maternally-inherited cardiomyopathy (Tables **1** and **4**) [33, 36].

The obtained results of the research prove the data of Russian scientists that in case of using various methods of investigation a different range of mutations can be observed. However the method of Next-Generation Sequencing allows detecting a greater number of mutations associated with the investigated pathologies.

2. MITOCHONDRIAL GENOME MUTATIONS IN DIABETES

2.1. Mutations in mitochondrial diabetes

2.1.1. Italian scientists conducted whole mitochondrial genome sequencing of 11 patients with mitochondrial diabetes, their mothers and 80 healthy individuals [39]. Previously an enrichment of DNA samples from patients by mitochondrial genome molecules was performed by long PCR using GeneAmp PCR System 9700 (Applied-Biosystems, Foster City, CA, USA). Then an analysis of the whole mitochondrial genome was performed using the BigDye Terminator v3.1 cycle sequencing method on the ABI-Prism 3730 Genetic Analyzer (Applied-Biosystems). Only 33 mutations from 416 variants of mitochondrial DNA detected during this analysis were found in patients and their mothers. 22 of these mutations (67%) were localized in the coding region (Table **5**). Besides in one of the patients heteroplasmic mutation 3243A>G was found, which is usually associated with MIDD (maternally inherited diabetes and deafness). For mutation 3243A>G the level of heteroplasmy was detected using real-time quantitative PCR (qRT-PCR). For the rest of mutations, found in this study, the level of heteroplasmy was not calculated.

Therefore, major mutations of a coding region, associated with mitochondrial diabetes, are localized in complexes I, III, IV and V. Common mutations non-coding region, associated with mitochondrial diabetes, are localized in D-Loop and NC7 regions, and also in genes of RNRs and tRNAs.

Table 4. Mitochondrial genome mutations associated with mitochondrial cardiomyopathy.

Gene	Homoplasmic mutation		Heteroplasmic mutation	
	Detected by the Sanger method	Detected by the NGS method	Detected by the Sanger method	Detected by the NGS method
MT-RNR2	-	m.3010G>A	-	-
MT-TL1	-	-	-	m.3243A>G
	-	-	-	m.3645T>C
tRNA-Ser (MT-TS1)	-	-	-	m.7501T>C
MT-ATP8	m.8473T>C	-	-	-
MT-COX3	-	-	-	m.9854T>G
MT-ND5	-	m.13967C>T	-	-
MT-CYTB	-	-	-	m.15222A>G
	-	m.15326A>G	-	-
D-loop region	-	-	-	m.16093T>C
	m.16566G>A	-	-	-

Table 5. Common mitochondrial genome mutations associated with mitochondrial diabetes.

Localization in mitochondrial respiratory chain complex, RNA or a non-coding region	Gene	Mutation
Complex I	MT- ND1	m.4024A>G
		m.4086C>T
	MT- ND2	m.5093T>C
		m.5300C>T
	MT-ND3	m.10373G>A
	MT-ND4L	m.10685G>A
	MT-ND4	m.11253T>C
		m.11447G>A
		m.11928A>G
	MT-ND5	m.12346C>T
		m.13135G>A
		m.14002A>G
	MT-ND6	m.14365C>T
		m.14502T>C
		m.14582A>G
Complex III	MT-CYB	m.15530T>C
Complex IV	MT-COX2	m.7762G>A
	MT-COX3	m.9803A>G
		m.9935T>C

(Table 5) Contd….

Localization in mitochondrial respiratory chain complex, RNA or a non-coding region	Gene	Mutation
		m.9947G>A
		m.9548G>A
Complex V	MT-ATP8	m.8562C>G
tRNA	MT-TV	m.1664G>A
	MT-TL1	m.3243A>G
RNR	MT-RNR1	m.951G>A
		m.960delC
D-loop region	HVI ((Hypervariable segment 1)	m.16048G>A
		m.16137A>G
		m.16354C>T
		m.16526G>A
	HVII (Hypervariable segment 2)	m.293T>C
		m.385A>G
Non-coding nucleotides region	MT-NC7 (non-coding nucleotides region 7)	8289_8290insCCCCCTCTA

It is necessary to mention that mutation 3243A>G (gene MT-TL1) was found to be associated with two risk factors of atherosclerosis: mitochondrial diabetes and mitochondrial cardiomyopathy.

2.1.2. In an investigation of researchers from the UK mitochondrial genome mutations m.12258C>A (tRNA-Ser gene) and 14709T>C (tRNA-Glu gene) were detected in two individuals in a sample of 28 patients with maternally inherited diabetes using the technology of NGS [40]. The investigation was carried out with the use of ABI Prism 377 DNA sequencer (Appied Biosystems).

It is necessary to note that in a research of Russian scientists, heteroplasmy of mitochondrial mutation in position 14709T>C was associated with atherosclerotic lesions of carotid arteries. It may indicate that there are common mechanisms in pathogenesis of atherosclerosis and maternally inherited diabetes [40, 41].

2.2. Mitochondrial Haplogroups Associated with Diabetic Complications

During an analysis of mitochondrial haplogroups, which included Sanger sequencing, Italian scientists detected mitochondrial genome haplogroups associated with diabetic complication [42]. It was found that mitochondrial haplogroup H was associated with diabetic cardiovascular complications and diabetic retinopathy. Besides, individuals having cardiovascular complications had a lower level of HDL cholesterol. Perhaps this decrease of level is associated with the presence of haplogroup H in patients. In addition, an association of mitochondrial haplogroups H3, U3 and V with diabetic nephropathy was detected.

It is noteworthy that Russian scientists in their investigation found an association of haplogroup U with atherosclerosis and left ventricular hypertrophy [34]. As diabetes and left ventricular hypertrophy are risk factors of atherosclerosis, the detected association of haplogroup U with diabetic nephropathy and left ventricular hypertrophy may indicate that there are common molecular pathogenetic mechanisms of these three pathologies.

CONCLUSION

With the use of the NGS technology it proved to be possible to detect a range of homoplasmic and heteroplasmic mutations and mitochondrial genome haplogroups which are associated with atherosclerosis and its risk factors. Meanwhile some mutations and haplogroups were detected both in atherosclerosis and in its risk factors, such as mitochondrial diabetes, mitochondrial cardiomyopathy, diabetic nephropathy and left ventricular hypertrophy. It conveys the suggestion that there are common pathogenetic mechanisms causing these pathologies.

What's next? Most of chronic pathologies in humans, including atherosclerosis, start long before the overt clinical manifestations occur. The time lapse for hidden progression of atherosclerosis takes years and decades. During this time, the person at risk and physician both remain unaware of the existing premorbid pathological condition. As a result, no preventive measures are undertaken to reduce the individual risk of the development of the overt disease. Genetic diagnostics of individual predisposition to atherosclerosis may provide a set of informative biomarkers to disguise the hidden but progressing pathology at the very early stages. However, the existing arsenal of pharmaceutical agents does not provide a legal room for physician to perform preventive intervention in the individual who is considered healthy at the present time. The conventional therapy is unjustified, since no symptoms, no targets, and no indications for active treatment exist. Therefore, the solution may be found in the field of complementary and alternative medicine. As a rule, complementary approaches allow for continuous and even lifetime management of subclinical atherosclerosis by the means of generally safe non-pharmaceutical agents, which are mainly of natural origin, and per se represent a step towards the development of true pharmaceutical agents. However, the solutions suggested by complementary and alternative medicines are often underestimated, and, therefore, they meet healthy skepticism but deserve thorough and peer evaluation by the experts. As a result, new paradigm of crosstalk between non-pharmaceutical and true

pharmaceutical approaches may be developed to fill the niche of effective and pathogenically targeted pretreatment and treatment of preclinical and subclinical atherosclerosis to avoid the development of chronic life-threatening disease.

DISCLOSURE STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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