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Lab Note

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Short-term serum deprivation causes no significant mitochondrial DNA mutation in vascular smooth muscle cells revealed by a new next generation sequencing technology

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Mitochondrion provides much of the energy for the cell and the disorder in mitochondrion can affect almost any tissue [1]. The mitochondrial DNA (mtDNA) covers 16,569 bp, consisting of 37 genes and 1 D-Loop region in human. Mitochondrion is unique in possessing their own genome which is organized into mitochondrial nucleoids by employing multiprotein machineries, and can perform the functions of gene replication, transcription, and repair [2]. Although it has almost all known nuclear DNA repair pathways, the lack of protective histones may lead to particular susceptibility to stresses and DNA damage events [3]. Indeed, the mtDNA has a very high mutation rate [4]. Since its discovery, mtDNA mutation has been known to be common and has emerged as an important cause of human diseases including diabetes, cardiovascular diseases, cancers, neuromuscular disorders, and aging, affecting 1 in 4300 of the population [5,6]. Thus, identifying mtDNA mutations in stresses and diseases is now a hot research area.

Vascular ischemia is a common phenomenon in many vessel-related disorders and diseases, such as diabetes, premature aging, cardiovascular disease, and cancers. Undoubtedly, ischemia is a strong stress for various types of cells. This stress can stimulate serum deprivation response factor [7], phosphatidylinositide 3-kinase (PI3K)/Akt pathway [8], and so on [9], thus having serious biological consequences. Therefore, ischemia may be a driving or worsening factor for these vascular diseases.

Currently, it is not known whether ischemia leads to mtDNA mutations in vascular diseases [10]. Smooth muscle cell (SMC) is a major contributor to the development of arterial disease. Cultured vascular SMCs are important for vascular disease research and can be used to identify new therapeutic targets for arterial diseases. Here, we first employed a new next generation sequencing (NGS) technology, VariantPro™ Capture Technology (VPCT), to identify the possible mutations of mtDNAs in human aortic smooth muscle cells (HASMCs) under serum deprivation condition.

VPCT is a completely new NGS platform based on a special targeted library, which is designed by LC Sciences (Hangzhou, China). This platform is designed to simplify and improve the targeted sequencing workflow. The whole VariantPro™ Mitochondria Panel, including specific primer design (OmegaPrimer™; **Supplementary Fig. S1**), VariantPro™ amplicon design (**Supplementary Fig. S2**), one-step library construction, unique polymerase chain reaction (PCR) design (RelayPCR™; **Supplementary Fig. S3**), and detailed bioinformatic analysis (**Supplementary Fig. S4**), can perform the capture of mtDNA with high efficiency. This protocol is a new mutiplex PCR-based technology which can overcome issues encountered

by conventional PCR-based methods, such as complicated workflow, long hands-on time, high equipment cost, sequence dropout, and low uniformity. Relatively, VPCT is a low-cost and highly efficient new technology for high-throughput mtDNA sequencing.

To identify possible mtDNA mutations, HASMC (ScienCell, Carlsbad, USA) was obtained from JENNIO Biological Technology (Guangzhou, China) and maintained in 10 mm dish with Dulbecco's modified Eagle's medium containing 4.5 g/l glucose, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified incubator at 37°C with 5% CO_2. When the cells reached 80%–90% confluence, cells were subject to serum deprivation for 5 h. The normally cultured and serum-deprived cells were both collected for DNA extraction. The total DNA from cultured cells was extracted using DNA extraction kit (Beijing ComWin Biotech Co., Beijing, China) according to the manufacturer's instructions. The quality and the quantity of six samples were evaluated and all the qualified samples were used to construct the DNA library. The main procedures of library construction were performed following the instructions of LC Sciences. In library construction, the PCR amplification was performed for 5–10 ng DNA using VariantPro™ primer pools. The amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, UK). Each library was diluted to 20 pM for high-throughput sequencing. The sequencing was performed with Hiseq 4000 (Illumina, San Diego, USA). At least 2 × 150 bp reads for two ends were carried out, that is, 150 bp reads for each end. The sequencing data were analyzed following the procedures provided by LC Sciences.

The total sequencing information is listed in **Supplementary Table S1**. The standard value of Q30 in Illumina must be more than 75%, and our Q30 is more than 94%, indicating that our sequencing quality is very confident. The sequenced results were blasted with the mitochondrial genome to screen possible mutations. Our results showed that the average coverages of all the amplicons were more than 20% of the mean values (**Supplementary Fig. S5**). The fold changes were all less than 10 for most (>86.83%) of the amplicons, indicating that our libraries are of high quality with good homogeneity. This laid a solid foundation for our further high-throughput sequencing, which ensures that the system works very well and the sequencing results are reliable. Based on the sequenced reads for the control group and the test group, we analyzed the single nucleotide polymorphism (SNP), the insertion, the deletion, and the related mutation frequency for all the mtDNA genes in the control and test samples. It was found that 3 genes have insertions or deletions and 12 genes have SNP. Although these mutations occurred both in the control and in the test samples, no significant difference was found between the control sample and the test sample (**Fig. 1**).

In our previous experiment, we found that energy change is related to serum deprivation (data not shown). Therefore, we thought that serum deprivation of HASMCs might cause mtDNA mutations. Here, we do not find any mtDNA mutation in cells with 5 h of serum deprivation, possibly suggesting that short-term (<5 h) ischemia may lead to only changes at mitochondrial RNAs or proteins levels but not cause mutations at mtDNA level. However, prolonged serum deprivation, e.g. 24 or 48 h, may cause mtDNA mutations, which should be confirmed by further investigations. We indeed detected mutations in mtDNA, but no difference was found between the control group and the test group, suggesting that the normal controls also have some mtDNA mutations. This result is very important for the investigation of mtDNA mutation. We should not use only the standard reference genome but the normal control for each experiment should also be sequenced for identifying mtDNA mutations. Furthermore, in gene expression study, cell transfection is a universal technique in which serum deprivation is a routine practice. Here, we did not find any mtDNA mutation in HASMCs which had been subject to short-term ischemia, implying that routine serum deprivation in cell transfection experiment is safe for gene expression investigations, and the protocol is reliable.

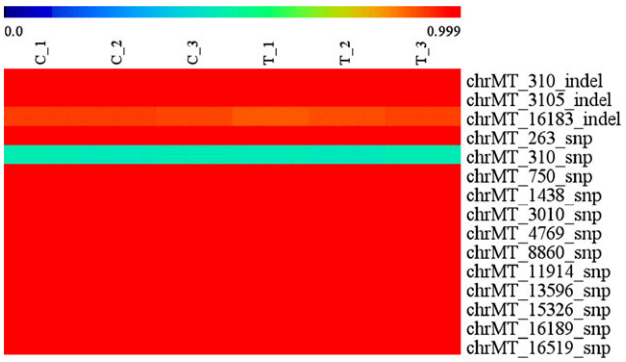

Figure 1. Mutation frequency of mtDNA genes Expression difference of the mtDNA genes quantitated by the normalized mutation frequency. Mutation frequencies are color-coded. The lowest mutation frequency is showed in blue, and the highest in red. Sample 'C' indicates the control group (normal cells), and 'T' indicates the test group (serum-deprived cells). C-1 to C-3 and T-1 to T-3 means three biological repetitions and independent sequencing for each group. Names of mtDNA genes are listed on the right. Snp means single nucleotide polymorphism and indel means insertion or deletion.

In summary, we introduced a new NGS technology to investigate mtDNA mutations in HASMCs under short time of serum deprivation. No significant difference was found in mtDNA mutations between normal cells and serum-deprived cells, implying that this new technology can be used for further investigating the biological effects of ischemia.

Supplementary Data

Supplementary data are available at *ABBS* online.

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