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Establishment of human retinal mitoscriptome gene expression signature for diabetic retinopathy using cadaver eyes

Gowthaman Govindarajan[a], Saumi Mathews[b], Karthik Srinivasan[c], Kim Ramasamy[c], Sundaresan Periasamy[a,*]

[a] Department of Genetics, Aravind Medical Research Foundation, Aravind Eye Hospital, Madurai, Tamil Nadu, India
[b] Department of Immunology and Stem Cell Biology, Aravind Medical Research Foundation, Aravind Eye Hospital, Madurai, Tamil Nadu, India
[c] Retina Clinic, Aravind Eye Hospital, Madurai, Tamil Nadu, India

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ABSTRACT

Diabetic retinopathy (DR) is a leading cause of blindness due to retinal microvasculature. We used microarray analysis for the first time to establish the retinal mitoscriptome gene expression signature for DR using human cadaver eyes. Among the 1042 genes, 60 (52-down, 8-up) and 39 (36-down, 3-up) genes were differentially expressed in the DR as compared to normal control and diabetic retinas respectively. These genes were mainly responsible for regulating angiogenesis, anti-oxidant defense mechanism, ATP production and apoptosis contributing to the disease pathology of DR. These findings might be useful for the discovery of biomarker and developing therapeutic regimen.

1. Introduction

Diabetic Retinopathy (DR) is a progressive microvascular complication caused by diabetes mellitus, which affects the structure and cellular composition of the retina eventually leading to blindness (Kuwabara and Cogan, 1963; Sims, 1986). Microaneurysm, macular edema, vessel dilation, blood-retinal barrier breakdown, vascular permeability, neovascularization, capillary non-perfusion, preretinal and vitreous hemorrhage are the clinical characteristic features of DR. Although multifactorial, the inheritance of DR can be attributed to the interaction of one or more genes due to the involvement of various genetic factors (Balasubramanyam et al., 2002; Awata et al., 2002; Balasubbu et al., 2010). Over the decade, several genetic studies were carried out in different ethnic groups and have identified candidate nuclear genes involved in the pathogenesis of DR (Saraswat et al., 2008; Fu et al., 2010; Huang et al., 2011). However, role of these candidate genes in the development of disease is poorly understood (Olga Simó-Servat et al., 2013).

Recently, a few studies were focused on exploring the role of mitochondria in the pathogenesis of DR (Kowluru and Abbas, 2003; Kowluru et al., 2006; Kofler et al., 2009). This shift in focus is probably due to the observation of dysfunctional retinal mitochondria with damaged mitochondrial DNA (mtDNA) and increased apoptosis in diabetic conditions (Kowluru, 2005; Podestà et al., 2000). Mitochondria are the major endogenous source of reactive oxygen species (ROS) such as superoxide, peroxynitrite and hydroxyl radicals (Kowluru et al., 2006). The most common ROS in the cell is superoxide radical, which is produced during oxidative phosphorylation within mitochondria (Kowluru et al., 2006). During oxidative stress, there is an increased production of ROS (Madsen-Bouterse et al., 2010; Manoli et al., 2005). This excess production originates from endogenous or exogenous sources or inefficient removal of ROS due to defect in the antioxidant defense system, causing pathological conditions (Graham et al., 2004; Kujoth et al., 2005; Jian et al., 2010). A related study has showed that oxidative stress as one of the important factors involved in the pathogenesis of DR (Petrovic et al., 2008).

Human mitochondrial DNA (mtDNA) encodes 13 proteins essential for the respiratory chain but the vast majorities (> 1000) of mitochondrial proteins, needed for mtDNA replication, translation, scaffolding and structure maintenance are synthesized by the nuclear DNA (Mootha et al., 2003; Graham et al., 2004; Kujoth et al., 2005). Highly integrated cross – functionality of these nuclear and mitochondrial genomes are essential for maintenance of cellular homeostasis. The transcriptomes of nuclear DNA and mtDNA can be together called mitoscriptome (Raju et al., 2011). The high relevance of mitochondria in the maintenance of cellular structure and function places mitoscriptome analysis as an important piece of the puzzle in understanding the pathogenesis of DR. Hence the present study was focused on developing a customized mitoscriptome based cDNA microarray for systematically understanding the role of mitochondria in disease pathogenesis of DR

* Corresponding author at: Department of Genetics, Aravind Medical Research Foundation, Aravind Eye Hospital, No.1 Anna Nagar, Madurai 625 020, Tamil Nadu, India.
E-mail address: sundar@aravind.org (S. Periasamy).

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Table 1
Human cadaver donor eye details such as age, sex and cause of death.

S.no	Study groups	Age (y)	Sex	Cause of death
1.	Control			
	RA-Id-380	75	Male	Chronic obstructive pulmonary disease
	RA-Id-382	82	Male	Heart attack
	RA-Id-383	82	Female	Respiratory diseases
	RA-Id-415	60	Male	Heart attack
2.	Diabetes			
	RA-Id-99	79	Male	Cardiac arrest
	RA-Id-101	71	Male	Myocardial infection
	RA-Id-105	65	Female	Ketosis
	RA-Id-109	85	Male	Natural
3.	Diabetic retinopathy			
	RA-Id-50	50	Female	Cardiac arrest
	RA-Id-237	74	Male	Stroke
	RA-Id-239	67	Female	Myocardial infection
	RA-Id-27	62	Male	Heart attack

using human cadaver eyes.

2. Materials and methods

2.1. Human tissue procurement

Human donor eyes were procured from Rotary Aravind International Eye Bank, Aravind Eye Hospital, Madurai within 1.9–3.0 h of death after obtaining written consent of the donor or the donor's family for use in medical research. All tissues were handled in accordance with the principles outlined in the Declaration of Helsinki and was approved by Aravind Medical Research Foundation Institutional Review Board. Other details such as sex, age, and cause of death, and a family report of a limited medical history were also documented carefully (Table 1). We used the technique developed by Decanini et al. (2008) for imaging the posterior pole to distinguish DR eyes. The fundus, including macula and optic nerve, were carefully examined for signs of DR.

The samples included in this study were categorized into three study groups – (Anderssohn et al., 2014) DR retina (Aslanukov et al., 2006) diabetic retina and (Awata et al., 2002) control retina. Whole globes with a medical history of diabetes and hallmark clinical features of DR such as microaneurysms, dot/blot hemorrhages or signs of hard exudates were categorized in to DR retina group (n = 4) (Fig. 1a). Whole globe with a medical history of diabetes but no signs of DR were included in the diabetic retina group (n = 4) (Fig. 1b). The normal retina with no history of diabetes and no evidence of retinal pathology were categorized into control retina group (n = 4) (Fig. 1c). Photographic documentation was carried out using Nikon cool pix 8400 8 MP.

Once after examination, neural retina were harvested within 24 to 48 h of enucleation and immersed in RNA later (Qiagen, Hilden, Germany) and stored at − 80 °C until further use.

2.2. Customization of human mitochip microarray

A custom based human mitochondria-focused gene chip was developed to understand the role of mitochondria in the development of DR. This human mitochondrial gene chip (mitochip) includes 1005 genes from the nuclear genome contributing to the mitochondrial structure and function as well as the 37 genes in the mtDNA (Table 2). Potential candidate genes were selected based on literature search using key words like mitochondrial biogenesis and oxidative stress in various online databases like NCBI (https://www.ncbi.nlm.nih.gov/), Mitocarta (www.mitocarta.org/html/database.html) and Mitoproteome (http://www.mitoproteome.org/) for developing human mitochip microarray. Transcriptional loci predicted from sequences were excluded from the mitochip. The probe sets were designed using Agilent custom algorithm and were printed on the 8 * 15 k Agilent platform. We deposited our customized human mitochip microarray in the Agilent database under the AMADID number: G2509F_045815.

2.3. RNA extraction and quantification from human neural retina

Total RNA was extracted from 100 mg of frozen neural retinal tissues using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, followed by DNase (Qiagen, Hilden, Germany) treatment. The concentration and purity of the RNA extracted were evaluated using the Nanodrop Spectrophotometer (Thermo Scientific 1000, USA) and tested for integrity on the Bioanalyzer (Agilent 2100, CA, USA). Samples that had an optimal RIN value of 6.0 and above were taken for microarray experiment.

2.4. RNA labeling, amplification and hybridization

The samples were labeled using Agilent Quick Amp Kit (Agilent Technologies, CA, USA). 500 ng of total RNA was reverse transcribed using oligodT primer tagged to T7 promoter sequence. cDNA obtained was converted to double stranded cDNA in the same reaction. Further, the cDNA was converted to cRNA in the in-vitro transcription step using T7 RNA polymerase enzyme and Cy3 dye was added into the reaction mix. During cRNA synthesis, Cy3 dye was incorporated into the newly synthesized strands. The resultant cRNA was cleaned up using Qiagen RNeasy columns (Qiagen, Hilden, Germany). Concentration and amount of dye incorporated was determined using Nanodrop. Samples that pass the quality control were taken for hybridization. 600 ng of labeled cRNA were hybridized on the array using the Gene Expression Hybridization kit (Agilent Technologies, CA, USA) in sure hybridization chambers (Agilent Technologies, CA, USA) for overnight at 65 °C. Hybridized slides were washed using Agilent Gene Expression wash buffers (Agilent Technologies, CA, USA). The hybridized, washed microarray slides were then scanned on a G4900DA scanner (Agilent Technologies, CA, USA).

2.5. Microarray data analysis

Images were quantified using Feature Extraction Software (Version-

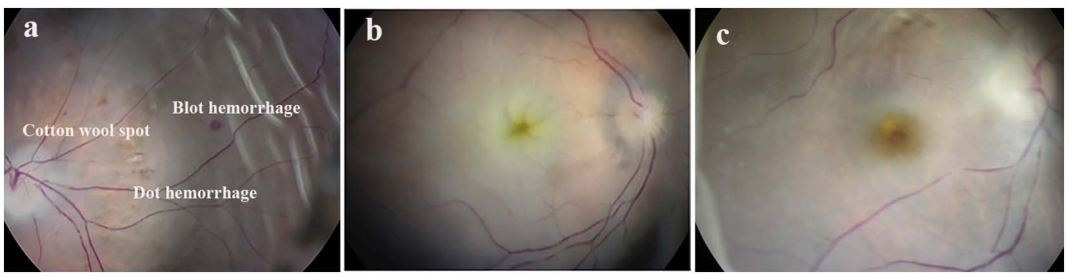

Fig. 1. Representative image of a human cadaver (a) DR retina with medical history of diabetes showing the clinical signs of DR such as cotton wool spot, blot and dot hemorrhage (b) diabetic retina without signs of retinopathy and (c) control retina. All the images were taken using Nikon cool pix 8 MP.

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