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Expression of *SVIL* gene in case of T2DR (Type 2 Diabetic Retinopathy) in Human Retinal Tissue, an Agent may have role in Neovascularization

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Abstract | **Original Research Article**

Healthy eyes are a deep subject in this modern world. There are several diseases that cause complication in vision and sometime cause blindness. Every eye disease disrupts the eye due to damage to different areas of eyes. Few of the ocular disease that acts on retina and cause massive damage to the eye. One of which is known as diabetic retinopathy that occurs in a patient suffering from diabetes mellitus due to excessive glucose stress in the eye. As a result of which, the retinal microvasculature that are responsible for proper blood flow in the four quadrants of the eyes and provide nutrients and proper oxygen supply to it and also act as a blood retinal barrier that protects the retina by excluding circulating molecular toxins, microorganisms, and pro-inflammatory leukocytes gets blocked. As a result of which the oxygen stress is created in the retina. It has been well known that due to the oxygen stress in the new blood vessels are formed in the retina but quite in a different way. The resulting new blood vessel formation is quite different from angiogenesis and known as neovascularization. Neovascularization is a key process for causing diabetic retinopathy in human eyes. At gene level in case of pathogenic condition of type 2 diabetic retinopathy, there is deregulation of various genes that are involved in causing pathogenicity. Actually there is a cascade of various events after which this condition arises. Using gene expression and epigenetic analysis, it has been possible to explore the expression profiling of various gene in the human retinal cell and contribute to understanding of the pathogenesis of these diseases. Additionally, it is possible to provide the support for the involvement of well-characterized biological molecules, and in this way, there is a chance to identify new players in retinal pathologies. It may lead to new to the design of new biological therapies.

Keywords: Type 2 Diabetic Retinopathy, *SVIL* (supervillin gene), Neovasularization, Retina, relative quantification.

INTRODUCTION

DIABETES, the most common disease among the several age groups is the fastest growing epidemic that has been caused by increased blood glucose level and body's incapacity to produce or regulate insulin1. It has been estimated that the number of diabetic patient will increase from 171 million people in 2000 to 366 million people by 2030 [1, 2]. Diabetes is a metabolic disorder that has been characterized by faulty secretion of insulin hormone which is secreted by the beta cells of pancreas. Type I diabetes has been characterized by the immune mediated destruction of beta cells of pancreas. While in a case of type II diabetes is characterized by insulin resistance and relative inadequacy in insulin signaling [1]. High blood glucose level is the major factor that is responsible for the development of diabetic complications [2].

Diabetes is that tragedy which will have a great impact on the lifestyle of people in the future, as well as the birth maker of many diseases. Diabetes is not a single disease but a bunch of several other complication and disease like diabetic neuropathy, diabetic nephropathy and many more. One of the complications that are related to human retina is known as diabetic retinopathy, the most extensive micro vascular complication of diabetes and it is the major cause of blindness all over the world. In a entire globe, there are 93 million people approximately has been suffering from diabetic Retinopathy, 17 million with proliferative DR, diabetic macular edema approx. 21 million people, and with VTDR approximately 28 million people [3].

Diseases that involves the retinal microvasculature, comprises of two ischemic vasculopathies i.e., diabetic retinopathy and retinopathy of prematurity and several other posterior forms of uveitis, are important reasons behind the blindness in both industrialized countries as well as the developing nations [4].

Diabetic retinopathy is not immediately onset with the onset of diabetes. But it has been proven that elevated blood pressure, poorer control of blood glucose and longer duration of diabetes are the most important factor for the onset of diabetic retinopathy. As per the Wisconsin Epidemiologic Study of Diabetic Retinopathy (WESDR) report in 1980-82, it had been showed that type I diabetic patient have prevalence of diabetic retinopathy approximately 17% in those who have diabetes less than 5 years and those who suffering more than 15 years, the chance of prevalence of diabetic retinopathy was almost 100%.) The corresponding statistics in type II diabetes patient were 29% and 78 % [5].

Supervillin is a member of the gelsolin superfamily of actin-binding proteins having molecular weight of 205 kDa [6]. Archvillin, muscle-specific isoform is a closely related 250 KDa protein [7]. Supervillin consist of sixgelsolin-related repeating units, plus an action-binding domain/nuclear localization signal [8]. Supervillin, one of the largest members of the villin/gelsolin family of actin-organizing proteins, that have both overlapping and distinct functions [6, 8, 9] Villin, concerned with precancerous morphological changes in epithelia, and gelsolin, concerned with the formation and degradative activity of osteoclast podosomes, and it is also promotes cancer cell invasion and motility [10-14].

Supervillin role in Invadopodial formation and help in cell motility suspects its role in neovascularization which is a major process in causing type II diabetic retinopthy. It has been suugested that high expression of supevillin is found in correspondonce with increased level of VEGF (main player of diabetic retinopathy) [15].

The word “epigenetic” literally meaning is that “in addition to changes in genetic sequence.” This term has been evolved to comprise any of the process that alters or changes the gene activity without changing the DNA sequence, and leads to certain modifications that can be transmitted to daughter cells [16].

There are several epigenetic processes that have been identified so far such as methylation, acetylation, phosphorylation, ubiquitylation, and sumolyation. These processes are natural and essential for organism functions, but improper function of this process can lead to major adverse health issues and behavioral effects [16].

The best known epigenetic process is DNA methylation which involves the addition or removal of methyl group from cytosine nitrogen base especially in the region where cytosine bases occur consecutively. DNA methylation has been observed in many illness and health conditions. Up regulation and down regulation of genes can be possibly occur due to the deregulation of epigenetic process. Therefore through methylation profiling of genes, we can predict the mechanism of the disease and it can provide more efficient gene target and new ways of therapeutics [16].

MATERIALS AND METHODOLOGY

Sample collection- Human retinal tissues (Donated) are collected from city prestigious institute Department of Ophthalmology, Gandhi Medical College (Bhopal). Proper care and handle must be taken for the entire provision for the collection of Retinal tisues and its transportation from these source station to our labs, School of biotechnology, RGPV Bhopal. Collection of donated human retinal endothelial cell has been approved by Institutional Ethics Committee for Human Research (IECH) VIDE letter no. 7824-26/MC/IEC/2016 dated on 22/03/2016.

Samples were collected in M-K medium (a media formulated by McCarey and Kaufman) that has been kept in triple layer casing filled with dry ice. For a prolonged storage, it was supplemented with 1% DMSO and kept in deep freezer between -40^{0}C to -80^{0}C.

Sample size includes 08 donors with 04 normal tissues, 02 donors having Type 2 Diabetes Mellitus, 02 donors having Type 2 Diabetic retinopathy.

Total RNA extraction and cDNA synthesis

Tissue RNA has been isolated from human retinal tissues by using RNA-Xpress reagent (MB601 MolBioHimedia) and performed as per the manufacturer’s protocol. Later the integrity was checked by running agarose gel (1.2%). Purity and concentration was checked by using Nanodrop ND-1000 spectrophotometer V 3.5.

The total RNA was reverse transcribed by oligo dT primers by using ProtoScript^R first strand cDNA Synthesis kit (BioLabs) in a thermal cycler **(**Gradient Palm Cycler from Corbett Life Sciences**)** and performed as per the manufacturer’s protocol. The reaction program was as follows. 70^{0}C for 5 min, followed by 42^{0}C for 60 min and 80^{0}C for 5 min, with a total volume of 20 µL which contained 150 ng of total RNA, 10 µL of M-MuLV Reaction Mix, 2 µL of M-MuLV Enzyme Mix, 2 µL of Oligo dT Primer(50 µM) , and DEPC treated water to make the volume 20µL.

(Prior to storage at -80^{0}C, the cDNA quality was evaluated by housekeeping gene (*GAPDH*) amplification, and then the reverse products were stored at -20 ^{0}C until use.

PCR Amplification and Relative Quantification

Later on gene specific PCR for *SVIL* has been performed by using oligo primers that has manually synthesized as shown in the table, which amplified the region of 165bp as available in the literature and optimum annealing temperature was optimized through Gradient PCR. PCR amplification program are as follow initial denaturation at 94^0 C for 3 min, and then 35 cycles of denaturation, annealing, and extension was performed (94^{0}C for 30s, 51^{0}C for 30 s and 72^{0}C for 30 s) and final extension at 72^{0}C for 5 min was done.

Relative quantification was done by $\Delta\Delta C_T$ method [17] in a Real Time PCR ((LightCycler^R 480 SYBR Green I Master from Roche Diagnostics).). Each sample was collected and each real time reaction was performed. For relative quantification by the comparative C_T method, values were expressed relative to a control sample (normal patient). The C_T for the SVIL gene and the C_T for the *GAPDH* (reference) were determined for each sample. The expression of SVIL gene were normalized by that of the reference gene, *GAPDH*, for all the samples and converted to the relative expression (fold of expression), as per following formula:

$$\text{Fold of expression (RQ)} = 2^{-\Delta\Delta C_T}$$

RESULTS

Total RNA were isolated from human retinal tissues and the bands were visualized by running agarose gel (1.2%). Two intact bands i.e. 28S and 18S were visible which shows the good quality RNA as shown in fig.1.

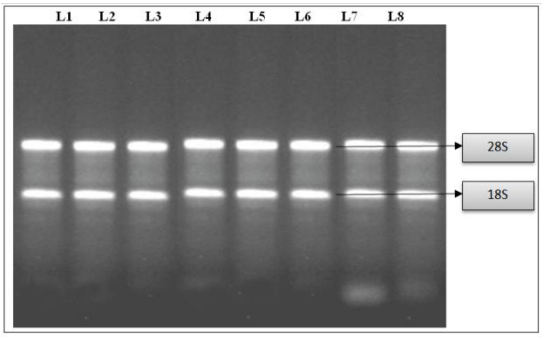

Fig-1: Agarose gel (1.2%) picture of RNA sample showing intact 28S and 18S rRNA bands

The reverse transcription PCR was performed by using oligo dT primers and further the integrity and genomic DNA contamination was checked by performing gene specific PCR for GAPDH. Amplified product of 240bp of GAPDH were visible by running PCR product along with 100bp DNA ladder on 2% agarose gel as shown on fig. 2.

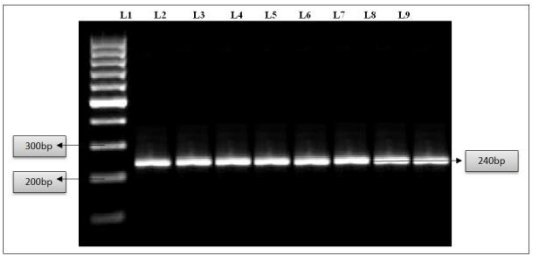

Fig-2: Agarose gel run (2%) of PCR product with *GAPDH* primers

Lane 1 (L1): 100 bp DNA Ladder

Lane 2 (L2-L9): 240bp amplified PCR product of *GAPDH* gene

Gene specific PCR for Supervillin gene with annealing temperature 50°C was performed for each sample through the manually synthesized oligo primers from cDNA prepared from Retinal RNA of human. On agarose gel electrophoresis of amplified PCR product of *SVIL* gene along with 100bp DNA ladder revealed a single specific band of 165bp.

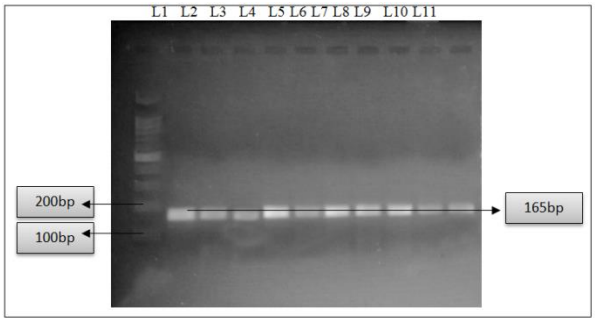

Fig.3 Agarose gel run (2%) of PCR product with *SVIL*primers for each sample (P6, P9 toP12)
Lane 1 (L1): 100 bp Ladder

Lane 2 (L2-L11): 165bp amplified PCR product of *SVIL*gene

Real time PCR analysis of gene *GAPDH* and *SVIL* expression in human retinal tissue was performed which shows amplification as shown in fig.4

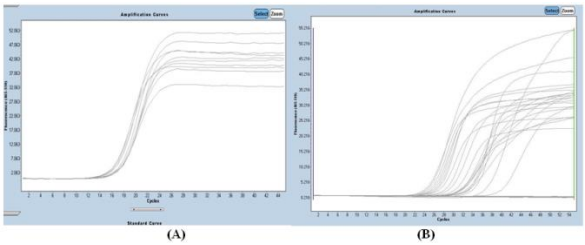

Fig-4: Amplification plot analysis of *SVIL* and *GAPDH* gene

- Amplification plot of *GAPDH*
- Amplification plot of *SVIL*

The relative quantification result showed that the higher expression of SVIL gene in case of T2DR in comparison of T2DM and normal retinal tissue where as little or negligible difference in the expression level of supervillin gene in case of T2DM and normal retinal tissue.

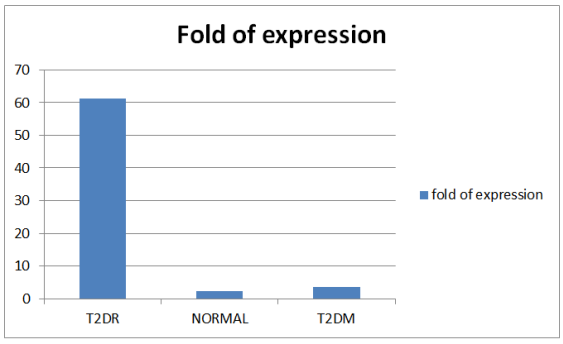

Fig-5: Fold of expression

T2DR – Type 2 Diabetic Retinopathy Retinal Sample
NORMAL- Normal Retinal Tissue
T2DM- Type 2 Diabetic Mellitus Retinal Sample

Table-1: Sequence of the primers which amplified the region of 240bp

Forward	A	G	C	T	G	A	A	C	G	G	G	A	A	G	C	T	C	A	C	T	G	G		
Reverse	G	G	A	G	T	G	G	G	T	G	T	C	G	C	T	G	T	T	G	A	A	G	T	C

Table-2: Sequence of the primers which amplified the region of 165bp

Forward	T	G	G	T	G	T	T	T	G	A	T	T	T	T	G	G	T	A	G	T	G	A	A	
Reverse	T	A	A	G	C	G	G	A	T	T	G	C	A	T	T	C	T	C	C	A				

CONCLUSION

Till today, the exact mechanism of the pathogenesis of this disease is not well understood. There are some key players that are responsible for causing this is never unfolding or remains underrated. The available method of treatment whether it is photocoagulation method, anti VEGF blockade, steroids have certain limitation in regards with the cure of this disease.

As of the available literature till now, there are some key holes that have to be studied in a sequence to understand the pattern of this disease and give a way for the ultimate cure of the disease.

In the present study, total RNA from the retinal tissue has been successfully isolated, and the cDNA synthesis with the oligo dT primer has also been done successfully. In order to confirm the quality of a cDNA, PCR amplification of *GAPDH* gene has also been done with the help of oligo primers that shows good quality cDNA and free from genomic DNA contamination. Expression of *SVIL* in human retinal tissues has been confirmed by normal PCR as well as with Real time PCR. All the samples taken *viz* healthy person, type II Diabetes Mellitus and human suffering from type II Diabetic Retinopathy displayed positive amplification of 165bp PCR product specific for *SVIL* gene. Higher expression of SVIL gene in case T2DR in comparison to T2DM and normal retinal tissues shows that its might be responsible for the incidence of T2DR and its role in neovascularisation confirms its presences in the pathogen city of T2DR. Further analysis must be done to confirms its exact role via proteomic analysis or through epigenetic.

As *SVIL* belongs to the gelsolin superfamily of actin binding protein [6] whose main function include formation of invadopaodia (similar as podosomes) [18-20, 17], as it is well known fact that in cancerous cell, supervillin help in cell invasion by the formation of invadopodia [21] Studies in HeLa cells show that supervillin promotes cell motility [22]. Reports of supervillin involvement in cell motility suggested a potential role in retinal vessel formation [15]. Similarly it may help endothelial cell for the undirected formation of blood vessels through the process of neovascularization. It is also proven that Cap G another member of the same family have role in endothelial cell motility [23]. Villin is associated with precancerous morphological changes in epithelia, and gelsoin in required for the formation and degradative activity of osteoclast podosomes; gelsolin also promotes cancercell invasion and motility [10, 14, 11-13]. So supervillin may have role in T2DR by helping in neovascularization. Supervillin in normal patient may involve in normal and directed formation of blood vessels through process of angiogenesis. And further role confirm by expression profile of *SVIL* gene in case of T2DR which includes large no. of samples.

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