Original Research *Highlight article*

Comparative transcriptome analysis of endothelial progenitor cells of HbSS patients with and without proliferative retinopathy

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Impact Statement

Genes and pathways potentially associated with sickle cell retinopathy were identified through the transcriptome profile of circulating endothelial progenitor cells obtained from HbSS patients with and without retinopathy. The findings provide favorable evidence for further studies of the molecular mechanisms involved in proliferative sickle cell retinopathy.

Abstract

Among sickle cell anemia (SCA) complications, proliferative sickle cell retinopathy (PSCR) is one of the most important, being responsible for visual impairment in 10–20% of affected eyes. The aim of this study was to identify differentially expressed genes (DEGs) present in pathways that may be implicated in the pathophysiology of PSCR from the transcriptome profile analysis of endothelial progenitor cells. RNA-Seq was used to compare gene expression profile of circulating endothelial colony-forming cells (ECFCs) from HbSS patients with and without PSCR. Furthermore, functional enrichment analysis and protein–protein interaction (PPI) networks were performed to gain further insights into biological functions. The differential expression analysis identified 501 DEGs, when comparing the groups with and without PSCR. Furthermore, functional enrichment analysis showed associations of the DEGs in 200 biological processes. Among these, regulation of mitogen-activated protein (MAP) kinase activity, positive regulation of phosphatidylinositol 3-kinase (PI3K), and positive regulation of Signal Transducer and Activator of Transcription (STAT) receptor signaling pathway were observed. These pathways are associated with angiogenesis, cell migration, adhesion, differentiation, and proliferation, important processes involved in PSCR pathophysiology. Moreover, our results showed an over-expression of *VEGFC* (vascular endothelial growth factor–C) and *FLT1* (Fms-Related Receptor Tyrosine Kinase 1) genes, when comparing HbSS patients with and without PSCR. These results may indicate a possible association between VEGFC and FLT1 receptor, which may activate signaling pathways such as PI3K/AKT and MAPK/ERK and contribute to the mechanisms implicated in neovascularization. Thus, our findings contain preliminary results that may guide future studies in the field, since the molecular mechanisms of PSCR are still poorly understood.

Keywords: Sickle cell anemia, proliferative sickle cell retinopathy, differentially expressed genes, endothelial colony-forming cells

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Introduction

Sickle cell anemia (SCA) is one of the most frequent genetic diseases in the world population. It results from the substitution of just one nitrogenous base, adenine, to thymine (GAG → GTG), in the seventh codon of the β-globin gene, leading to the substitution of glutamic acid with valine, and originating an abnormal hemoglobin, denominated hemoglobin S (HbS).[1,2]

Vaso-occlusion is the central process of the pathophysiology of SCA, although chronic anemia, hemolysis, and vasculopathy also contribute to a remarkable diversity of clinical complications. Vaso-occlusion and tissue ischemia are complex processes that involve an association of several factors such as inflammation, activation of endothelial cells, presence of procoagulant and pro-angiogenic molecules, oxidative stress, and vasculopathy.[3] The activated endothelium induces endothelial adhesion molecule expression,

contributing to the obstruction of the microvasculature and decreased blood flow. In addition, low levels of nitric oxide (NO) bioavailability, due to NO consumption by cell-free Hb, released during intravascular hemolysis, contribute to vasoconstriction and adhesion events.[4]

Among the ophthalmological changes present in individuals with SCA, retinopathy stands out as the most important, especially in its proliferative form (proliferative sickle cell retinopathy [PSCR]), representing the major cause of progressive vision loss in these patients, and compromising 10–20% of affected eyes.[5,6] PSCR is triggered by vaso-occlusion of the microvasculature in the peripheral retina, resulting in tissue ischemia and neovascularization. Retinal neovascularization is promoted by angiogenic mediators released due to repeated episodes of arteriolar occlusion and ischemia. New vessels grow at the posterior border of the non-perfusion zone and are characterized by flat vessels that take a frond-like form of denominated sea fan structures, the hallmark of PSCR.[7] Sea fans often spontaneously regress (20–60%) due to autoinfarction or neovascularization and can progress leading to vitreous hemorrhage, retinal ruptures and rhegmatogenous or tractional retinal detachments, which compromise vision.[7–9]

Studies using animal models have revealed several aspects of the molecular pathogenesis of retinal neovascularization. The mechanism of retinal neovascularization is triggered by over-expression of hypoxia inducible factor–1 (HIF-1) under conditions of hypoxia. This over-expression leads to increased levels of hypoxia-regulated genes, such as vascular endothelial growth factor (VEGF), one of the most relevant angiogenic factors in neovascularization and in the inflammatory response.[10]

Endothelial colony-forming cells (ECFCs), also known as blood outgrowth endothelial cells, late outgrowth endothelial cells, or late endothelial progenitor cells (EPCs), represent an endothelial cell type (endothelial lineage) that exhibits clonal potential, with potent angiogenic capacity that leads to vascular formation *in vitro* and *in vivo*.[11–13] ECFCs can be isolated from peripheral blood and umbilical cord, which are characterized by an immunophenotype described as positive for the endothelial markers, CD31, CD146, VEGFR2, and von Willebrand's factor; negative for myeloid markers CD45 and CD14; and negative or low for endothelial activating antigen, CD34.[11] Studies have demonstrated that ECFCs are recruited into ischemic retina or damaged outer retina and help to promote vascular repair and revascularization of ischemic retinopathies.[14–16]

The purpose of the present study was to identify differentially expressed genes (DEGs) in ECFCs from HbSS patients with PSCR that may be associated with this ocular complication. We compared the gene expression profiles of ECFCs from patients with SCA and proliferative retinopathy versus patients without retinopathy using RNA sequencing (RNA-Seq). In order to integrate transcriptomic and proteomic data, Gene Ontology (GO) and Protein–Protein Interaction (PPI) network analysis from RNA-Seq data were performed. The data shown in the present study may contribute to subsequent studies of the retina and confirm the remarkable diversity of angiogenic signaling pathways involved in PSCR, thus providing many opportunities for therapeutic intervention.

Materials and methods

Patients

After cautious analysis of medical records, five patients with the HbSS genotype were selected from the Hematology and Hemotherapy Center, University of Campinas-UNICAMP (Campinas, São Paulo, Brazil). Among the group, three had PSCR (Group 1) and two had no evidence of ophthalmic pathological signs (Group 2). The diagnosis of SCA was confirmed by clinical and laboratory data. Supplementary Tables 1 and 2 show the demographic characteristics of patients, along with their hematological parameters, clinical data, and information regarding drug therapy. The peripheral blood samples were obtained after ophthalmological analysis, followed by immediate culture of ECFCs. In the case of patients undergoing regular blood transfusion (patients 1 and 5), blood samples were collected immediately before receiving the red blood cells. Exclusion criteria were malignancy, diabetes *mellitus*, painful crisis within two weeks before the time of ocular examination, pregnancy, ocular media opacities that prevented fundoscopic examination, hydroxycarbamide therapy, and previous eye surgery.

This study received approval by the University Ethics Committee based on national guidelines, and all patients agreed to enroll by signing written informed consent.

Ophthalmologic evaluation

Complete ophthalmic examination was performed for all patients included in this study; the exams included anterior biomicroscopy, fundus biomicroscopy using 78-diopter lens, indirect fundoscopy with a 20-diopter lens, color fundus photography, and fluorescein angiography. The same retinal ophthalmologist from the Department of Ophthalmology at the State University of Campinas examined all patients. For Group 1 (PSCR), patients were diagnosed in at least one of the stages according to Goldberg's classification[17]: neovascularized sea fan at the boundary between the non-vascularized and vascularized zones of the retinal periphery (Stage III), presence of vitreous hemorrhage (Stage IV), and tractional or rhegmatogenous retinal detachment (Stage V).

ECFCs culture

To perform ECFC culture, peripheral blood samples of five HbSS patients were obtained, according to methods previously described.[12] Approximately 45 mL of peripheral blood were collected in 9 mL of sodium heparin. In summary, phosphate buffered saline (PBS) was added to anticoagulated blood in a 2:1 ratio; this diluted blood was layered over an equivalent volume of Ficoll-Paque PLUS (GE Healthcare) before centrifuging at 317 *g* for 30 min at room temperature. Subsequently, mononuclear cells were separated and washed thrice with EBM-2 medium (Lonza, Walkersville, MD, USA) and resuspended in EBM-2 medium (Lonza, Walkersville, MD, USA) containing EGM-2 (Growth Medium endothelial cells–2) BulletKit™, 10% additional fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), and 1% L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA).

Approximately 7×10^6 cells were seeded in 12-well flat-bottom tissue culture plates precoated with type 1 rat tail collagen (Sigma-Aldrich, Saint Louis, MO, USA), and stored in a humidified incubator with 5% CO_2. After 1–3 weeks of incubation, ECFCs were isolated and identified by their characteristic cobblestone morphology and evaluated for surface marker expression using a FACSCalibur flow cytometer (BD Bioscience, San Jose, CA, USA). FlowJo software (Tree Star Inc.) was used to analyze the results. ECFCs were verified by positive staining for the endothelial markers CD31, CD144, CD146, VEGF/KDR; negative or low staining for endothelial activating antigens, CD34; negative for the myeloid cell marker, CD45, and for the endothelial progenitor marker, CD133.

RNA extraction

After reaching 80–90% confluence, 0.025% trypsin-EDTA was used to harvest ECFCs. Trizol Reagent (Ambion Life Technologies, Carlsbad, CA, USA) and a commercial RNeasy mini-kit (Qiagen GmbH, Hilden, Germany) were used to extract total RNA from ECFCs according to the manufacturer's protocol. To remove the remaining genomic DNA, the RNA was treated with DNAse I (Life Technologies, Carlsbad, CA, USA). The NanoDrop 2000 spectrophotometer (Thermo Scientific, Carlsbad, CA, USA) was used to measure the quantity and purity of the extracted RNA, and the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA) was used to determine the RNA integrity.

RNA sequencing and differential gene expression analysis

RNA-Seq was carried out with ECFC samples from Groups 1 and 2, comprising a total of five patients. RNA libraries were prepared using Illumina TruSeq RNA-Seq v2 kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Libraries were submitted to the Agilent Bioanalyzer (Agilent, Santa Clara, CA) for quality control and subjected to high-throughput 100 bp paired-end sequencing on a HiSeq 2500 sequencer. The FastQC v0.11.5 program[18] was used to assess read quality, and the STAR v2.5.2 program[19] was used to read alignments based on the human reference genome (GRCh38.88). Only those reads mapped exclusively with the reference genome were considered for downstream analysis.

To generate count matrices from the reads aligned to the genome, the *featureCounts* function of Rsubread R package was used.[20] Normalization and detection of differential gene expression were obtained with the DESeq2 R package.[21] The $\log_2$ fold change score and $\log_{10}$ *P* values were used to build a Volcano plot in EnhancedVolcano package v1.2.0.[22] A significant difference in gene expression levels was considered when $|\log_2 \text{ fold change}| \geq 2$, along with a false discovery rate (FDR) lower than 0.05. In order to visualize the overall differential gene expression levels, heatmaps were built using ClustVis online tool (https://biit.cs.ut.ee/clustvis/) with Pearson's correlation coefficient.[23]

GO and PPI network analysis

The PANTHER v14.1 online tool (http://www.pantherdb.org/) was used to perform the GO functional enrichment analysis by statistical over-representation for biological processes. All expressed genes identified in the RNA-Seq with counts > 5 were used to formulate the reference list, and all DEGs were included in the list of analyzed genes. Statistical analysis was performed using Fisher's exact test; biological processes with FDR < 0.05 were considered statistically significant.

To build and visualize the PPI networks, the Ensembl IDs of the DEGs were used in the stringApp plug-in present in the Cytoscape Network Analysis software v3.8.1 (https://cytoscape.org/). The parameters set were confidence score cutoff = 0.9 and maximum additional interactions = 0 followed by functional enrichment analysis using genome as background and filtering only GO biological process results. In addition, to identify protein clusters from the main network of protein interaction, generated through STRING, we used Molecular Complex Detection (MCODE) with the following parameters: degree cutoff of 2, node score cutoff of 0.2, haircut true, fluff false, and a K-score of 2.[24]

Results

After sequencing, each transcriptome produced an average of 70 million reads per 101 base-pair paired-end. GC content was approximately 49%. The quality score of 30 (Q30) was achieved in a minimum of 88% of the bases read, thus indicating a low sequencing error rate of 1:10.000. Furthermore, the data are considered to be of high quality, with 85–94% of the reads mapped exclusively to the human genome.

Identification of DEGs

Those genes that obtained FDR < 0.05 and $|\log_2 \text{ fold change}| \geq 2$ were established as statistically significant. Thus, 501 DEGs were identified when comparing groups 1 and 2. Among these DEGs, 235 (45.9%) were downregulated and 266 (53.1%) were up-regulated. To visualize the difference in expression between the groups, heatmap graphic was constructed (Figure 1). In addition, the principal components

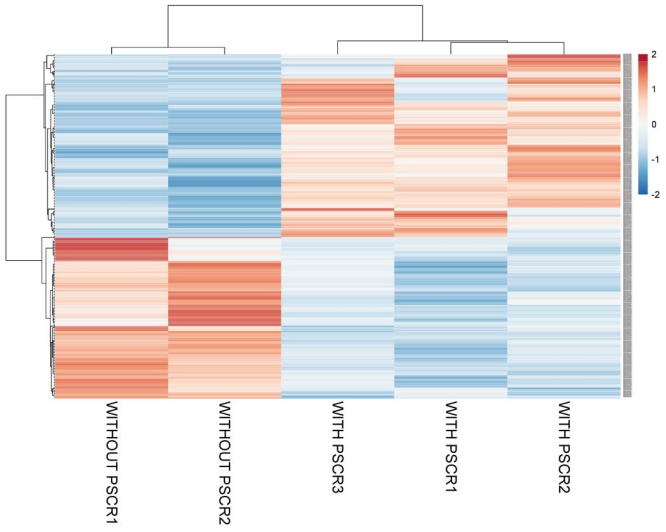

Figure 1. Heatmap graph generated from 501 DEGs identified by RNA-Seq after comparing patients with ($n = 3$) and without ($n = 2$) PSCR.
DEG: differentially expressed gene; PSCR: proliferative sickle cell retinopathy.

Table 1. GO terms identified from the analysis of functional enrichment of biological processes resulting from the comparison of patients with and without PSCR.

GO ID	Biological processes	DEGs	FDR
GO:0045765	Regulation of angiogenesis	22	8.50E-04
GO:0030155	Regulation of cell adhesion	47	5.86E-05
GO:0030334	Regulation of cell migration	46	4.23E-04
GO:0042127	Regulation of cell population proliferation	61	8.58E-03
GO:0043405	Regulation of map kinase activity	14	4.25e-03
GO:0014068	Positive regulation of phosphatidylinositol 3-kinase signaling	9	3.75E-02
GO:1904894	Positive regulation of receptor signaling pathway via STAT	6	4.90E-02

GO: Gene Ontology; PSCR: proliferative sickle cell retinopathy; DEG: differentially expressed gene; FDR: false discovery rate; STAT: Signal Transducer and Activator of Transcription.

analysis (PCA) of the transcripts shows the formation of distinct groups between patients with and without PSCR (Supplementary Figure 1).

Functional enrichment and PPI network analysis

The PANTHER GO functional enrichment by over-representation analysis found 200 significant biological processes, with 157 over-represented and 43 under-represented (Supplementary Table 3). Significantly enriched GO terms were associated with regulation of angiogenesis (GO:0045765; FDR = 8.50e-04), regulation of cell adhesion (GO:0030155; FDR = 5.86e-05), regulation of cell migration (GO:0030334; FDR = 4.23e-04), regulation of cell population proliferation (GO:0042127; FDR = 8.58e-03), positive regulation of phosphatidylinositol 3-kinase (PI3K) (GO:0014068; FDR = 3.75e-02), regulation of map kinase activity (GO:0043405; FDR = 4.25e-03), and positive regulation of receptor signaling pathway via Signal Transducer and Activator of Transcription (STAT) (GO:1904894; FDR = 4.90e-02) (Table 1). In Supplementary Table 4, the GO terms mentioned above and the respectively associated DEGs are displayed.

In order to better understand the PPIs involved, all 501 DEGs identified from previous analysis of RNA-Seq data were used to feed stringApp, a Cytoscape software plug-in (https://cytoscape.org/). After filtering the unconnected nodes, the main network was generated containing 97 proteins with 81 interactions between them (Supplementary Figure 2). In addition, the MCODE plug-in was used to build clusters based on the main network. A total of five clusters were generated, with three protein nodes or more (Figure 2).

Discussion

Regarding ophthalmological alterations present in SCA, retinopathy is considered the most important, especially in its proliferative form. The diagnosis of PSCR is based on the presence of neovascularization, which occurs in response to vascular occlusions and retinal ischemia/hypoxia. In this scenario, EPCs are recruited from the bone marrow and enter the systemic circulation. There is evidence that the recruitment of EPCs and their consequent differentiation into mature endothelial cells would be an additional mechanism in the recovery of vascular integrity and angiogenesis. Currently, important studies use these cells as a therapeutic target in the treatment of ischemic retinopathy and have shown their high proliferative and neovascularization capacity.[25,26]

ECFCs have a high proliferative capacity[11–13] and have been used as a model for the pathophysiological study of vascular disease[27] and as a source of cells in the process of vascular regeneration.[28,29] Several significant studies have used ECFCs to stimulate vascular repair and revascularization of ischemic retinopathies, such as diabetic retinopathy and retinopathy of prematurity. Thus, these cells have become accessible indicators of vascular status, allowing the investigation of vascular pathologies during the preclinical stages.[30]

There are few studies in the literature that relate PSCR to differential gene expression.[31] Hence, the gene expression profile of ECFCs from patients with SCA and proliferative retinopathy versus patients without retinopathy using RNA-Seq were compared. Our data showed genes and pathways with over-representation in biological processes associated with angiogenesis, cell migration, cell adhesion, and cell proliferation. These processes are expected since the PSCR pathophysiology is directly related to these mechanisms, which can be triggered by the presence of vaso-occlusion, hypoxia, ischemia, tissue damage, and neovascularization.[6]

Interestingly, three GO terms that describe different biological processes were identified, which can contribute to the pathophysiological mechanisms of PSCR: GO:0043405, which describes the regulation of mitogen-activated protein (MAP) kinase activity; GO:1904894, which describes the positive regulation of receptor signaling pathway via STAT; and GO:0014068, which describes the positive regulation of PI3K. These pathways play a key role in supporting the transmission of signals from receptor tyrosine kinases (RTKs) to the nucleus.[32–34]

MAP kinases are enzymes present in MAPK cascades. In most cells, MAPKs are part of a group of cytoplasmic enzymes that transmit mitogenic and cell differentiation signals.[35] Activation of these enzymes occurs in response to a variety of extracellular mechanisms, such as lipopolysaccharides, hypoxia, release of inflammatory cytokines, and growth factors, such as epidermal growth factor (EGF) and epidermal growth factor receptor (EGFR). The binding of EGF to its RTK, EGFR, leads to its dimerization and activation, causing activation of the MAPK pathway.[36,37] Activation of the MAPK pathway mobilizes a signaling cascade that culminates in the transcription of genes that encode proteins implicated in the regulation of important cell functions, such as cell proliferation, cell growth, and differentiation, inflammation, and apoptosis.[38–40] The canonical MAPK cascade is composed of three successive serine/threonine kinases:

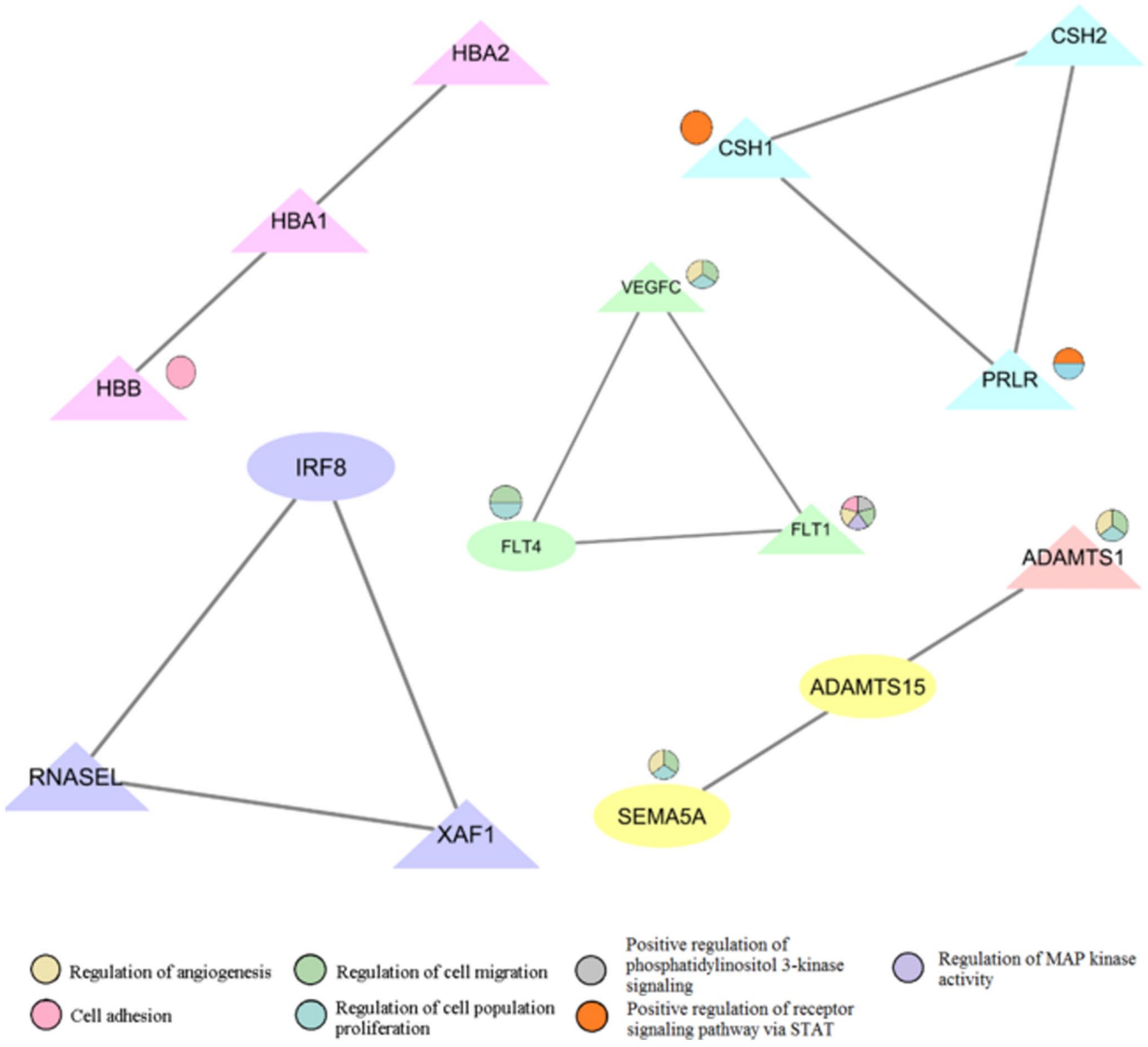

Figure 2. Five clusters of the intersection network identified by the MCODE (Molecular Complex Detection) plug-in from Cytoscape, derived from the main network of interactions between protein and protein. The nodes represent proteins and the edges represent protein interactions. The colors of the clusters were randomly selected. Triangles are proteins encoded by over-expressed genes, while circles represent proteins encoded by under-expressed genes. The pie charts indicate the main biological processes identified by Gene Ontology functional enrichment analysis (PANTHER online tool), which may contribute to the pathophysiological mechanisms of PSCR and respective associated proteins. The proportions shown in the pie charts do not reflect the actual percentages of the processes. PSCR: proliferative sickle cell retinopathy.

MAPK kinase kinase (MAPKKK), MAPK kinase (MAPKK), and MAPK.[41] MAPKKK itself is regulated in response to small GTPases, such as Ras.[42] The main components of this pathway are Ras, Raf, and MEK, which induce ERK activation, subsequently activating several transcription factors and cytoplasmic proteins that influence cell proliferation and survival.[31]

In addition, EGF binding to EGFR can also induce receptor dimerization and autophosphorylation of its intracellular domain, leading to activation of the STAT pathway. Members of the STAT pathway can act through the RAB3C/IL6/STAT3 pathway, in which GTPase RAB isoform 3C modulates the IL6/STAT3 pathway. It is suggested that over-expression of *RAB3C* in colon cancer cells facilitates cancer metastasis through promotion of IL-6 secretion and recruitment of members of the STAT3-related pathway. IL-6 regulates many cellular processes, such as migration, proliferation, and differentiation.[43] Furthermore, IL-6 has been shown to contribute to the development of cervical cancer through JAK-3/STAT6 signaling (Janus Kinase-3/signal transducer and activator of the STAT6 signaling pathway).[33] Interestingly, *RAB3C*, *IL-6*, and *STAT6* are up-regulated in RNA-Seq analysis when the groups with and without PSCR are compared. These data may indicate that signaling pathways including such genes may be associated with important pathways that participate in the development of PSCR, such as proliferation, migration, and angiogenesis. However, further studies are needed to confirm this hypothesis.

Furthermore, the PI3K pathway consists of ligands such as kinases, phosphatases, and transcriptional factors that act in intracellular signaling cascades essential for modulating processes of proliferation, migration, survival, and angiogenesis, being identified as a pattern of "classic" signaling.[34] In endothelial cells, these can be activated by different classes of membrane receptors, such as G protein-coupled receptors, tyrosine kinases, integrins, and apoptosis receptors.[44,45] This pathway is implicated in endothelial cell functions, including regulation of vascular tone, angiogenesis, cell adhesion, and recruitment of leukocytes to the vessel wall, with some PI3K isoforms playing an important role in NO release and recruitment of EPCs.[45]

In addition, PPI networks were performed. Among the resulting proteins, we highlight the VEGFC, Fms-Related Receptor Tyrosine Kinase 1 (FLT1), and FLT4 proteins. The human VEGF family contains five different encoding genes: *VEGFA*, *VEGFB*, *VEGFC*, *VEGFD*, and *PLGF* (placenta growth factor).[46] VEGFA, VEGFB, and VEGFC are blood vessel growth regulators and VEGFC and VEGFD preferentially regulate lymphatic angiogenesis.

VEGF interacts with endothelial cells through RTKs, VEGFR1 (fms-like tyrosine kinase 1 or Flt-1), VEGFR2 (fetal liver kinase, FLK1, or KDR), and VEGFR3 (fms-like tyrosine kinase 4, FLT4). VEGFA interacts with VEGFR1/FLT1 and VEGFR2/FLK1, while PlGF and VEGFB bind to the VEGFR1/FLT1 receptor. VEGFC and VEGFD predominantly bind to VEGFR2/FLK1 and VEGFR3/FLT4.[47] The binding of VEGF to its different receptors leads to the activation of different intracellular signaling pathways involved in the angiogenesis mechanisms.[48,49] Furthermore, VEGF like PLGF and Stromal-derived factor-1 (SDF-1) promotes recruitment of EPCs into ischemic or injured tissue and stimulates neovascularization.[50–52]

Retinal hypoxia and consequent tissue ischemia induced by recurrent vaso-occlusions in sickle cell disease, stimulate the production of pro-angiogenic factors, such as the VEGF factor, promoting the pathological proliferation of blood vessels, leading to the neovascularization of the retina. This abnormal neovascularization grows in the vitreous cavity and can cause retinal detachment by traction. Studies have suggested that VEGF-C signaling, perhaps through interactions with VEGFR-1/FLT1, may perform an important role in the development of acute myeloid leukemia with changes related to myelodysplasia.[53]

Interestingly, the analysis of RNA-Seq data showed an over-expression of *VEGFC* and *FLT1* genes and under-expression of *FLT4* when comparing the groups. These results may indicate a possible association between *VEGFC* and *FLT1* receptors, thereby activating signaling pathways and contributing to the mechanisms involved in angiogenesis and in the pathophysiology of sickle cell retinopathy. Furthermore, inhibition of endothelial cell apoptosis has been considered an essential mechanism during angiogenesis. Solovey *et al*.[54] reported higher levels of VEGF in the plasma of sickle cell patients and an inverse correlation between VEGF levels and the percentage of circulating endothelial cells in apoptosis. The authors suggested that a state of increased anti-apoptotic tone for endothelial cells in sickle cell patients may be related to the pathophysiology of the disease, particularly in the development of proliferative retinopathy. Indeed, increased VEGF levels in the retina of HbSS patients with PSCR have been reported.[55]

Curiously, the binding of VEGF to its different receptors leads to the activation of different intracellular signaling pathways. Briefly, this binding leads to autophosphorylation of receptor tyrosine residues, resulting in the activation of signal transduction molecules such as PI3K, protein kinase B (AKT/PKB), Ras, and MAPK among others. Activation of these molecules stimulates the PI3K and MAP kinase pathways leading to proliferation, migration, cell adhesion, and angiogenesis.[48,49] Thus, these pathways can interact with each other, forming a complex signaling network in mammals, with more than one possible gene/pathway interaction.[37]

Although the present work has some limitations, such as the small number of samples, the wide spectrum of clinical manifestations in SCA patients, which hampers an isolated assessment of PSCR, and the lack of quantification of the mRNA and protein levels of DEGs, our results indicate that cell migration, cell adhesion, and proliferation are important processes involved in PSCR. However, further studies are needed to confirm these data.

With regard to treatment, two patients (one patient with PSCR and one patient from the control group) were undergoing regular blood transfusion. Red blood cell transfusion remains an important therapeutic intervention for many complications associated with sickle cell disease. The significant question concerns whether blood transfusion might have effects on gene expression in circulating ECFCs. We highlight that the peripheral blood samples for isolation of ECFCs were collected immediately before the transfusion of red blood cells. To support the design of our study, we cite the work of Milbauer *et al*.[56] The authors showed that gene expression changed significantly after ECFCs stimulation with TNF-α and IL1-β. However, expression of all transcripts returned to baseline within one subsequent expansion. In our study, we used ECFCs from passages 3–5 for RNA sequencing. Therefore, we believe that the effects of transfusion treatment may have been reduced after all these passages, and that the observed expression profile reflects only cell culture conditions and the genetic profile of the patients.

We further emphasize that, given the inherent difficulties in obtaining the human retina, our approach used cultured circulating ECFCs, which are not endothelial cells that naturally reside in the affected organ and, therefore, are free of tissue-specific genetic factors. The application of this approach, based on the use of cultured ECFC as endothelial reporter cells, followed by extensive preliminary validation studies, has previously been used in the exploration of other vascular diseases.[56] In addition, studies have shown that EPCs that prevail in the systemic circulation have the ability to migrate and differentiate into mature endothelial cells in the ischemic area and promote the formation of neovessels.[57] Furthermore, studies using mice as models have provided evidence that ECFCs may play an important role in the regeneration of retinal vasculature and in the reduction of pathological angiogenesis in a model of ischemic retinopathy.[14–16]

Therefore, our study, unprecedented as far as we know, presents preliminary data that provide new insights that may

guide important studies in this area, in which the molecular mechanisms of retinopathy are still poorly understood.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the study, and review of the manuscript; MTI and RCS participated in sample collection; MGMV conducted the ophthalmological evaluation; SMSC, MTI, RCS, DMA, and CL conducted the cell isolation and culture; VHB, SMSC, MTI, RCS, JNPC, and TARR conducted the review of medical records; BBS conducted the bioinformatic and statistical analysis; VHB, SMSC, VMR, and MBM conducted the interpretation of transcriptome data; VHB, SMSC, PRSC, and JNPC conducted the Gene Ontology analysis; VHB and TARR conducted the protein–protein interaction analysis; SMSC, FFC, and MBM supervised the whole work; VHB, SMSC, and MBM wrote the manuscript; VHB and SMSC participated in an equivalent way.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

This study was approved by the Research Ethics Committees of the Faculty of Medical Sciences, UNICAMP, in accordance with national guidelines (protocol no. 3.960.683) and Declaration of Helsinki.

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SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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