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ORIGINAL ARTICLE

Comparative transcriptomic analysis of circulating endothelial cells in sickle cell stroke

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Abstract

Ischemic stroke (IS) is one of the most impairing complications of sickle cell anemia (SCA), responsible for 20% of mortality in patients. Rheological alterations, adhesive properties of sickle reticulocytes, leukocyte adhesion, inflammation and endothelial dysfunction are related to the vasculopathy observed prior to ischemic events. The role of the vascular endothelium in this complex cascade of mechanisms is emphasized, as well as in the process of ischemia-induced repair and neovascularization. The aim of the present study was to perform a comparative transcriptomic analysis of endothelial colony-forming cells (ECFCs) from SCA patients with and without IS. Next, to gain further insights of the biological relevance of differentially expressed genes (DEGs), functional enrichment analysis, protein–protein interaction network (PPI) construction and in silico prediction of regulatory factors were performed. Among the 2469 DEGs, genes related to cell proliferation (*AKT1*, *E2F1*, *CDCA5*, *EGFL7*), migration (*AKT1*, *HRAS*), angiogenesis (*AKT1*, *EGFL7*) and defense response pathways (*HRAS*, *IRF3*, *TGFB1*), important endothelial cell molecular mechanisms in post ischemia repair were identified. Despite the severity of IS in SCA, widely accepted molecular targets are still lacking, especially related to stroke outcome. The comparative analysis of the gene expression profile of ECFCs from IS patients versus controls seems to indicate that there is a persistent angiogenic process even after a long time this complication has occurred. Thus, this is an original study which may lead to new insights into the molecular basis of SCA stroke and contribute to a better understanding of the role of endothelial cells in stroke recovery.

Keywords Sickle cell anemia · Ischemic stroke · Endothelial colony-forming cells · RNA-Seq

Introduction

Sickle cell anemia (SCA), a hemoglobinopathy of monogenic inheritance, is a severe disease with an annual incidence of 300,000 births worldwide. A homozygous single point mutation in the seventh codon of the beta-globin gene (c.20A > T) leads to the replacement of glutamic acid for valine (p.Glu7Val), with the formation of HbS, a hemoglobin variant. Erythrocyte sickling due to HbS polymerization is the primary event in the complex pathophysiology of sickle cell disease, with consequent hemolysis, endothelial dysfunction and vaso-occlusive phenomena [1].

Stroke is one of the most impairing complications of SCA, and among the cerebrovascular alterations, ischemic stroke (IS) is the classic syndrome, responsible for 20% of mortality in patients [2, 3]. Features of sickle cell disease

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that predispose patients to the vasculopathy include rheological alterations, adhesive properties of sickle reticulocytes, leukocyte adhesion, endothelial dysfunction, inflammation and biomechanical disturbances from fluid shear stresses generated by increased blood flow secondary to anemia [4].

Predisposition to stroke is associated with altered blood flow velocity in large arteries related to excessive intravascular hemolysis in SCA, which can be detected pre-symptomatically by Transcranial Doppler Ultrasound (TDU). TDU is the standard method to assess IS risk; an elevated blood flow velocity (≥ 200 cm/s) is related to a higher risk [5, 6]. In these cases, blood transfusions can reduce the occurrence of IS in 90% [7]. Other approaches for IS prevention and treatment are based on hydroxyurea and anticoagulant therapies, but with no confirmed efficacy [8, 9].

Clinical and pre-clinical studies have identified Endothelial Progenitor Cells (EPCs) as a potential therapeutic target, as well as a positive correlation between the presence of these cells and functional recovery [10–12]. In cerebral ischemia, these cells are recruited from the bone marrow to the infarcted areas to repair the affected tissue through vascular remodeling, angiogenesis and secretion of growth factors, vasodilatory molecules and anti-inflammatory cytokines, which can promote the protection of the neurological vascular unit [13–15]. Endothelial Colony Forming Cells (ECFCs) are a type of EPCs that differentiates into mature endothelial cells, with high proliferative capacity and a stable phenotype during in vitro culture [16]. ECFCs play a pivotal role in vaso-occlusion and in post-ischemia recovery. These cells participate in neovascularization and secrete angiogenic factors, being a relevant study model in ischemic conditions [17–19].

Several approaches have been used to evaluate the genetic factors involved in IS. Candidate gene analysis, genome wide association studies and exome sequencing have found single nucleotide variants (SNVs) in genes related to inflammation, neurotransmission and signaling pathways [20–22]. However, these methodologies require a large sample size, and the lack of replication in other cohorts is frequent [23]. Unlike the genome, which remains unaltered through life, the transcriptome and proteome are dynamic. Transcriptomic analysis provides direct information about cell and tissue-specific gene expression characteristics, which is essential for a better understanding of the cellular and tissue metabolism and for assessing whether and how alterations in transcriptome profiles affect health and disease [24]. In this context, a previous study has identified, through microarray analysis, differentially expressed genes associated with inflammatory, cell adhesion, lipid metabolism and angiogenic pathways in patients with SCA and IS. Nevertheless, this technique is limited to evaluating only a subset of genes [17].

Despite the efforts on elucidating the genetic mechanisms underlying IS, few of them are related to stroke as a complication of SCA. Thus, given this scenario, our aim was to identify differentially expressed genes in ECFCs involved in sickle cell stroke using bulk RNA-Sequencing (RNA-Seq), a comprehensive method with the capability to capture basically the whole set of mRNA with high coverage. Our approach was based on the comparison of the gene expression profile of ECFCs from SCA patients with IS versus SCA patients without IS. Next, to gain further insight on the molecular mechanisms and biological relevance of the differentially expressed genes (DEGs), we have performed functional enrichment, protein–protein interaction network (PPI) construction and in silico prediction of regulatory factors. This original transcriptomic analysis enables a more comprehensive view of ECFC's gene expression alterations in response to IS, with exploratory results that may contribute to the identification of genes and molecular pathways as potential therapeutic and prognostic targets for this severe complication of SCA.

Casuistic and methods

Patients

This is a case–control study. All eight participants were recruited from the Hematology and Hemotherapy Center of the Universidade Estadual de Campinas (São Paulo, Brazil). The case group encompasses four patients (aged > 18 years) with SCA and history of clinical primary ischemic stroke (IS) confirmed by magnetic resonance imaging (MRI). In the control group four patients with SCA (aged > 20 years) and absence of clinical IS or silent infarcts confirmed by MRI were included. The imaging diagnosis was performed by a neurologist from the Neuroimaging Laboratory, Department of Neurology at the Universidade Estadual de Campinas. The exclusion criteria were patients with MRI evidence of hemorrhagic stroke, presence of other hemoglobinopathies such as HbC, HbD and β-Thalassemia or hereditary persistence of fetal hemoglobin. No patient was in hydroxycarbamide therapy. In the case of patients undergoing transfusion treatment, blood collection was performed before the procedure, at least 15 days after the previous transfusion. This study was approved by the University ethics committee, and written informed consent was obtained from each participant.

MRI acquisition and evaluation

MRIs were acquired and evaluated according to Ito et al. (2020)[17]. Briefly, a 3-T Philips Intera Achieva scanner was used with six different sequences, including magnetic

resonance angiography. An experient neurologist revised all scans, along with routine radiological evaluation. Besides the identification of major structural lesions, related to acute or remote stroke lesions, signs of cerebral microangiopathy or microvascular disease were also evaluated.

Demographic and hematologic data are shown in Table 1, and SCA-related complications, treatment and MRI results are displayed in Table 2.

ECFC culture

Peripheral blood samples (45 mL) of eight HbSS patients were collected into sodium heparin (9 mL) for ECFC culture, as previously described[25, 26]. In summary, phosphate buffer saline (PBS) was added in a 2:1 ratio to the anticoagulated blood. Next, the diluted blood was layered over a Ficoll-Paque PLUS (GE Healthcare) in an equivalent volume for centrifugation at 317 g for 30 min at room temperature. Mononuclear cells were isolated and resuspended in EBM-2 medium (Lonza, Walkersville, MD, USA), with EGM-2 BulletKit (Lonza), 10% additional fetal bovine serum (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Gibco, Life Technologies). A 12-well flat-bottom tissue culture plate pre-coated with type 1 rat tail collagen (Sigma-Aldrich, Saint Louis, MO, USA) was used for cell seeding (7×10^6cells). The culture was held in a humidified incubator at 5% CO_2. Colonies of endothelial cells appeared between 10 and 21 days of culture. Cells from passages 3 to 5 were used for this study. ECFCs were identified by their typical cobblestone morphology and

Table 1 Hematological parameters of HbSS patients with and without IS

L	Sex	Age at sample collection	Age IS	RBC (10^6/µL)	Hb (g/dL)	WBC (10^9/L)	Reticulocytes (AN)	Platelets (10^9/L)	HbF (%)	HbS (%)	α-Thalassemia (α3.7)
With IS											
1	F	26	23	2.8	8.9	11.2	16.1	473	1.3	24.1	Absent
2	F	28	11	3.6	11.4	9.4	14.5	436	2.8	41.3	Absent
3	M	43	39	2.8	8.5	12.6	6.2	371	1.4	14.4	Absent
4	F	42	41	3.0	8.9	5.1	6.2	304	8.0	56.8	Heterozygous
Without IS											
5	F	34	-	2.7	7.4	8.6	11.2	420	3.6	88.0	Heterozygous
6	M	39	-	3.0	9.6	11.1	9.5	285	9.5	84.5	Absent
7	F	29	-	3.7	9.2	6.1	16.9	383	3.1	90.3	Heterozygous
8	F	29	-	2.3	7.9	8.2	12.4	433	14.8	79.2	Absent

F female, *M* male, *RBC* red blood cells, *Hb* hemoglobin, *WBC* white blood cells, *AN* absolute number, *HbF* fetal hemoglobin, *HbS* hemoglobin S

Table 2 Clinical data, treatment and MRI results of HbSS patients with and without IS

Patients	Clinical data	Treatment	MRI results
1	IS, cholecystectomy, leg ulcer	Transfusions, folic acid, desferasirox, CaCO3	Lacunar infarcts and signs of small vessel disease
2	IS, cholecystectomy, leg ulcer, retinopathy	Transfusions, folic acid, desferasirox	Lacunar infarcts and signs of small vessel disease
3	IS, cholecystectomy, leg ulcer, retinopathy, osteoporosis, membranoproliferative glomerulonephritis	Transfusions, folic acid, desferasirox, enalapril maleate, CaCO3, alendronate sodium, acetyl-salicylic acid	Signs of small vessel disease
4	IS, cholecystectomy, osteoporosis, pulmonary hypertension, systolic hypertension, cardiac valve replacement	Transfusions, folic acid, enalapril maleate, $CaCO_3$, vitamin D, alendronate sodium, warfarin	Lacunar infarcts and area of gliosis in the left frontal lobe. Signs of small vessel disease
5	Retinopathy	Folic acid	No evidence of small vessel disease
6	Erectile dysfunction	Acetyl-salicylic acid	No evidence of small vessel disease
7	Cholecystectomy, retinopathy, pulmonary hypertension, hypothyroidism	Folic acid, enalapril maleate, levothyroxine sodium	No evidence of small vessel disease
8	No complications	Folic acid	No evidence of small vessel disease

MRI: Magnetic Resonance Imaging

characterized positively for endothelial markers CD31, CD144, CD146 and VEGF (vascular endothelial growth factor)/KDR (kinase insert domain receptor) but negatively or low for endothelial activation antigens and CD34 and negatively for the myeloid cell marker CD45 and the endothelial progenitor marker CD133. Flow cytometric analysis was performed using a FACSCalibur flow cytometer and BD FACSDiva 7.0 software (BD Biosciences).

RNA extraction

ECFCs with 80–90% confluence were harvested with 0.025% trypsin–EDTA. Total RNA extraction was performed by Trizol Reagent (Ambion Life Technologies, Carlsbad CA, USA) and a commercial RNeasy mini Kit (Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. To remove genomic DNA, RNA was treated with DNAse I (Life Technologies, Carlsbad, CA, USA). Nanodrop 2000 spectrophotometer (Thermo Scientific) was used for RNA quantification and purity evaluation. RNA integrity was determined by the Bioanalyzer 2100 System (Agilent Technologies).

RNA sequencing and differential gene expression analysis

RNA samples with RNA Integrity (RIN) > 7 were selected. Libraries were constructed using Illumina TruSeq RNA-Seq v2 kit (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Next, high throughput paired-end 100pb sequencing was performed on a HiSeq 2500 (Illumina, San Diego, CA, EUA) platform.

After sequencing, read quality was assessed by FastQC v0.11.9 (RRID:SCR_014583)[27]. Read alignment and assembly were performed using STAR v2.5.2 software (RRID:SCR_004463) [28] based on the human genome (GRCh38.88) as reference. Only the reads mapped exclusively to the reference genome were selected for the next steps. The *featurecounts* function of *Rsubread* [29] package in RStudio (RRID:SCR_000432)[30] was used to obtain a matrix of read counts for each gene and sample. Normalization and differential expression analysis were performed in the *edgeR* package [31] in RStudio. The cutoffs for differentially expressed genes (DEGs) were log2 fold change >|2| and false discovery rate (FDR) < 0.01. For graphic visualization of data, the packages *pheatmap* (Heatmap) [32], *PCAtools* (Principal Component Analysis) [33] and *EnhancedVolcano* (Volcano plot) [34] of RStudio were used.

Gene Ontology (GO) and protein–protein interaction network (PPI) analysis

DAVID v2021 online tool (RRID:SCR_001881)[35] was used for GO functional enrichment analysis of statistical over-representation for biological processes. All expressed genes obtained by the exact test function of *edgeR* were selected for the reference list. Statistical analysis was performed using Fisher's exact test, and biological processes with FDR < 0.05 were considered statistically significant.

To elucidate interactions between DEGs, genes contained in statistically significant biological processes were selected for PPI construction in *stringApp* plug-in [36] of Cytoscape Network Analysis Software v3.8.1 (RRID:SCR_003032) [37]. The main PPI network was built with the Ensembl ID of the selected genes as input in the *stringApp* plug-in, with confidence cutoff = 0.9. Next, the *cytoHubba* plug-in [38] was applied to the main PPI network to calculate the different scores for protein nodes and identify the top 5 hub-genes. The parameters for *cytoHubba* analysis were hubba nodes = 5 ranked by Degree with adjacent protein visualization. To illustrate the relation between hub-genes and biological processes, an alluvial plot was constructed in the SankeyMATIC tool[39].

In silico prediction of regulatory factors

To identify possible regulatory factors of the differential gene expression profile, in silico prediction of transcription factors (TFs) was performed. The identification of potential TFs was taken in *eXpression2Kinases (X2K)* web tool (RRID:SCR_016307) [40]. All the upregulated DEGs were selected as input, and the TFs were filtered by $p < 0.05$, with posterior selection of the TFs with upregulated hub-genes as targets. SankeyMATIC and Cytoscape were used to depict interactions between FTs-hub-genes.

Results

RNA-Seq and differential gene expression analysis

In this study, RNA-Seq was performed in ECFCs cultivated from SCA patients with ($n = 4$) and without ($n = 4$) IS. After sequencing, each library produced an average of 70 million reads with approximately 49% GC content. At least 88% of bases reached a Q-score of Q30, related to a base calling accuracy of 99.9%. Furthermore, 85–94% of reads were mapped exclusively to the human genome, indicating high quality data.

After normalization and differential gene expression analysis, 2469 genes were identified as differentially expressed (FDR < 0.01 and Log2FC >|2|). Of them, 1836 were upregulated (74.36%), and 633 were downregulated (25.64%). A distinct clustering between groups with and without IS is shown in Principal Component Analysis (PCA) (Fig. 1), in which the presence or absence of IS explains 86.36% of total group variance. This distinction

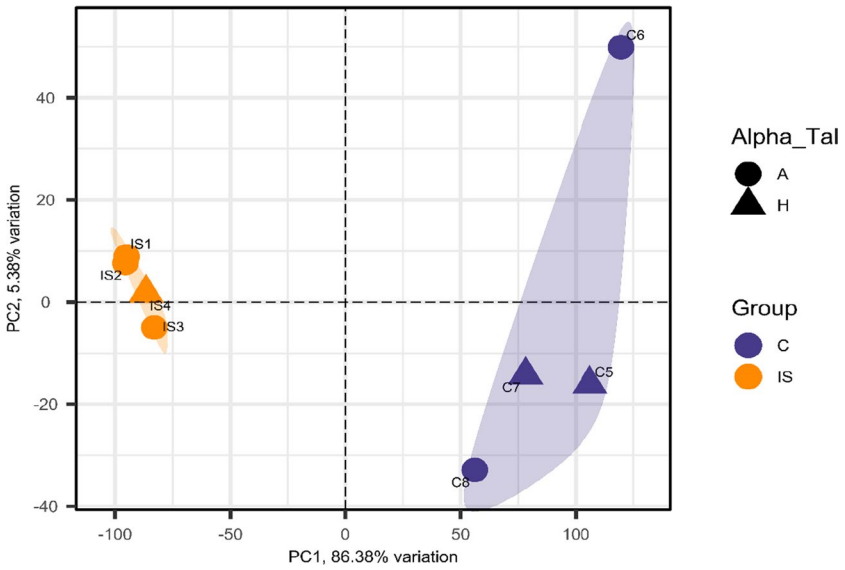

Fig. 1 Principal Component Analysis (PCA) of patients obtained from the DEGs. Principal Component 1 (PC1) represents 86.38% of group variation, and Principal Component 2 (PC2), 5.38%. The triangular shape corresponds to individuals heterozygous for α-thalassemia. C control group, IS ischemic stroke group, A absent, H heterozygous

is also present in Heatmap, with a differential gene expression pattern between the two groups (Fig. 2). The Volcano plot graph shows the distribution of differentially expressed genes (DEGs) regarding the up or downregulation profile (Fig. 3).

Gene ontology and PPI network analysis

The DAVID software was used for the functional enrichment analysis by over-representation of DEGs. There were 16 statistically significant biological processes, such as "defense response" (GO:0006952, FDR = 2.6E − 02); "inflammatory response" (GO:0006954, FDR = 3.9E − 02); "chemotaxis"

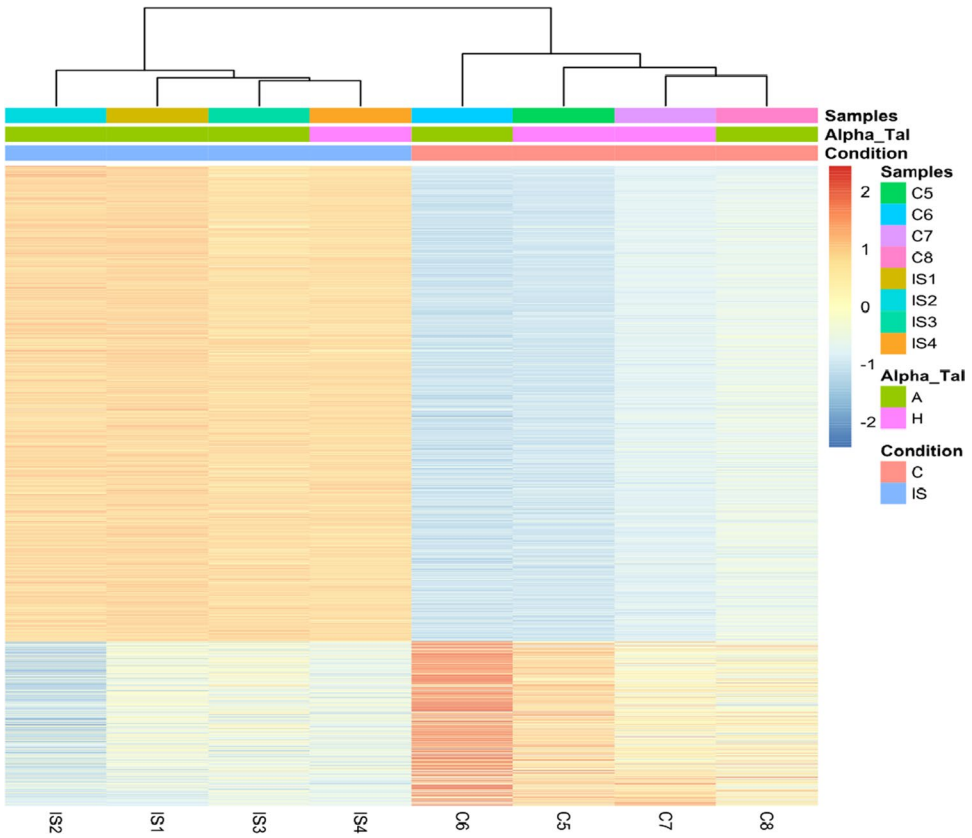

Fig. 2 Heatmap of differentially expressed genes. Rows indicate genes with significant differences in expression between the two groups; columns represent individual samples. Color intensity is proportional to gene expression. Upregulated genes are in orange, and downregulated genes in blue. C control group, IS ischemic stroke group, A absent, H heterozygous. Clustering method: Euclidean distance

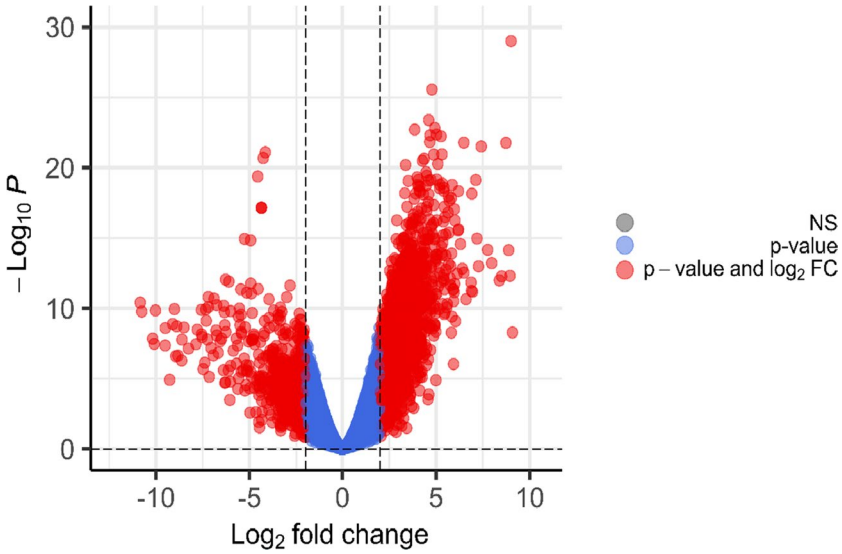

Fig. 3 *Volcano plot* of ischemic stroke (IS) versus control (C) comparison. X axis corresponds to Log2FC, and Y axis to − log10(FDR). DEGs are represented by red dots. NS not significant

(GO:0006935, FDR = 4.6E − 02) and "cell–cell adhesion" (GO:0098609, FDR = 4.6E − 02) (Fig. 4).

To better understand the biological relation between DEGs, a PPI network was constructed from the genes contained in the 16 gene ontology terms. The main network was constructed in the stringApp plug-in of Cytoscape, with 1519 proteins and 1556 interactions. Additionally, *cytohubba* plug-in was applied in the main network to identify the top 5 hub-genes. There were 137 proteins with 332 interactions. The top 1 hub-gene is *AKT1* (*AKT Serine/Threonine Kinase 1*, Log2FC = 2.57; FDR = 2.66E − 08); followed by *HRAS* (*HRas Proto-Oncogene, GTPase*, Log2FC = 3.71; FDR = 4.69E − 09); *PIK3R1* (*Phosphoinositide-3-Kinase Regulatory Subunit 1*, Log2FC = − 2.13; FDR = 3.92E − 05); *CDC20* (*Cell Division Cycle 20*, Log2FC = 2.62; FDR = 1.71E − 04) and *MAPK11* (*Mitogen- Activated Protein Kinase 11*, Log2FC = 2.71; FDR = 3.72E − 09) (Fig. 5). The relation between hub-gene and biological process is depicted in the alluvial plot (Fig. 6).

In addition to the hub-genes, other relevant genes are present in the total list of DEGs. They codify proteins that are present in mechanisms and molecular pathways associated to the biological context: *CDCA5* (*Cell Division Cycle Associated 5*, Log2FC = 2.22; FDR = 9.48E − 05) *EGFL7* (*EGF Like Domain Multiple 7*, Log2FC = 5.27; FDR = 5.8E − 23), *TGFB1* (*Transforming Growth Factor Beta 1*, Log2FC = 2.08; FDR = 1.02E − 07) all upregulated.

Regulatory factors

To identify possible regulatory factors of the gene expression profile of the PPI network hub-genes, in silico prediction of transcription factors was performed. For Transcription Factor Enrichment Analysis (TFEA), all the upregulated genes were selected. Next, only the statistically significant TFs with the four upregulated hub-genes as targets were selected.

There were 46 statistically significant TFs (Online Resource 1). Among them, *E2F1* (*E2F Transcription Factor 1*) has *AKT1* as target. For *HRAS*, seven TFs were found: *NFYB (Nuclear Transcription Factor Y* Subunit Beta), *PBX3* (*PBX Homeobox 3*), *IRF3* (*Interferon Regulatory Factor*

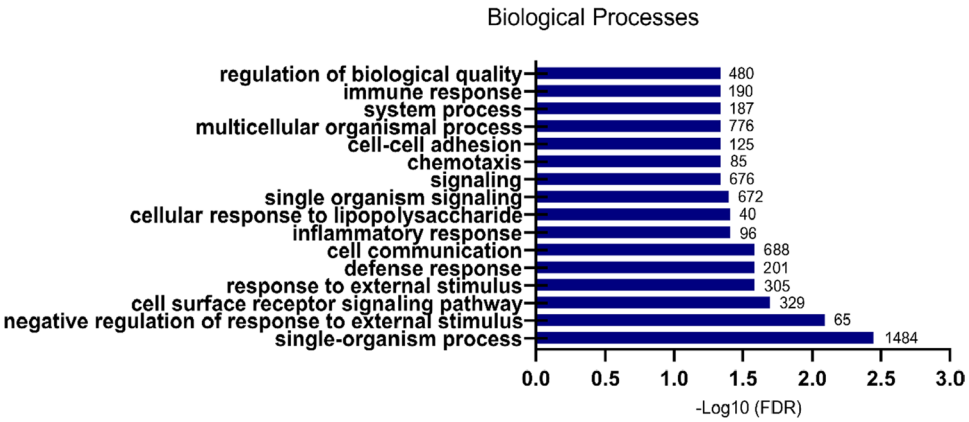

Fig. 4 Bar graph of the statistically significant biological processes. X axis corresponds to − Log10 (FDR), and Y axis to the gene ontology terms. The number in front of the bar is related to the number of DEGs in each biological process

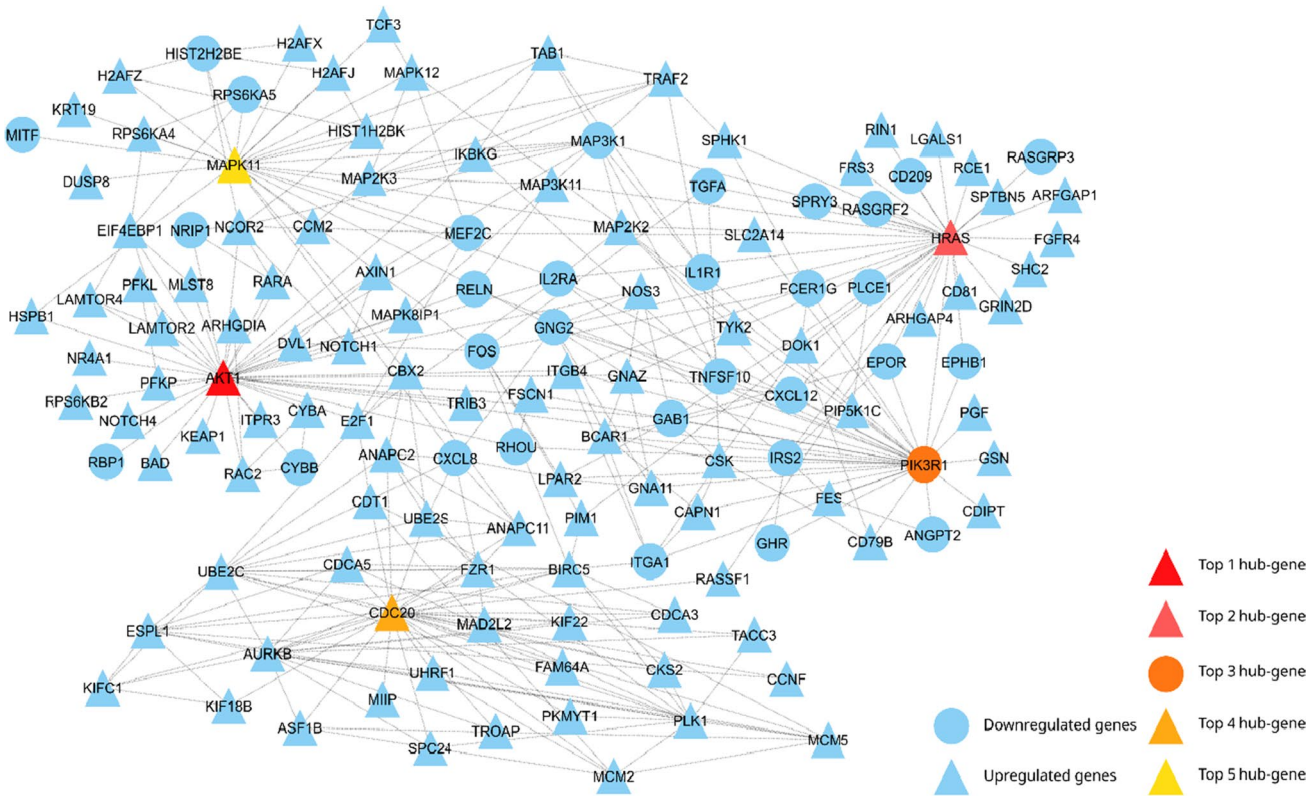

Fig. 5 PPI network of top 5 hub-genes: *AKT1*, *HRAS*, *PIK3R1*, *CDC20*, *MAPK11* and its interactions. Triangle-shaped nodes represent proteins encoded by upregulated genes, while round nodes represent proteins encoded by downregulated genes

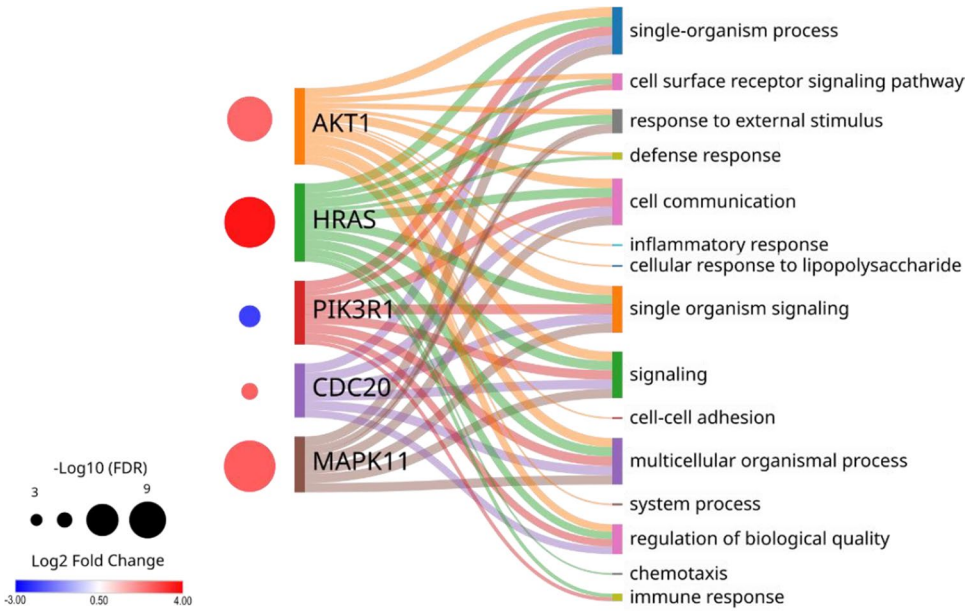

Fig. 6 Alluvial plot of hub-genes and correspondent biological processes. Circle size on the left of each gene corresponds to its RNA-Seq value of − Log10 (FDR), and the color to the Log2 Fold Change: upregulated genes are in red and downregulated genes in blue

3), *NFYA* (*Nuclear Transcription Factor Y Subunit Alpha*), *SP2* (*Sp2 Transcription Factor*), *GABPA* (*GA Binding Protein Transcription Factor Subunit Alpha*) and *BHLHE40* (*Basic Helix-Loop- Helix Family Member E40*). No TFs were identified for *MAPK11*. Finally, the *CDC20* gene is a potential target for six TFs: *NFYB (Nuclear Transcription Factor Y Subunit Beta*), *NFYA* (*Nuclear Transcription Factor Y Subunit Alpha*), *MAX (MYC Associated Factor X*),

BRCA1 (*BRCA1 DNA Repair Associated*), *FOXM1 (Forkhead Box M1*) and *E2F4* (*E2F Transcription Factor 4*). Interestingly, two TFs are upregulated in the present RNA-Seq: *E2F1* (Log2FC = 3.17; FDR = 1.78E − 07) and *IRF3* (Log2FC = 2.43; FDR = 3.11E − 09) (Fig. 7).

Discussion

Endothelial cell activation is an important mechanism in SCA complex pathophysiology, and in tissue recovery from ischemic events. Studies have identified the occurrence of neovascularization in humans in response to cerebral hypoxia [10], and the presence of Endothelial Progenitor Cells (EPCs) in this process in animal models [41]. ECFCs are circulating EPCs and have been used as a study model in vascular diseases, [25] including sickle cell anemia [17]. This is the first study to apply RNA Seq-based transcriptomic analysis to evaluate the gene expression profile of endothelial cells in IS, a severe complication of SCA. Through identification of differentially expressed genes, functional enrichment, PPI network construction and prediction of regulatory factors, we have identified genes associated with signaling (*AKT1*, *HRAS*, *PI3KR1*), cell surface receptor signaling pathway (*AKT1*, *HRAS*, *PI3KR1*), cell communication (*AKT1*, *HRAS*, *PI3KR1*), cell proliferation (*AKT1*, *E2F1*, *CDCA5*, *EGFL7*), migration (*AKT1*, *HRAS*), angiogenesis (*AKT1*, *EGFL7*) and defense response pathways (*HRAS*, *IRF3*, *TGFB1*), important endothelial cell molecular mechanisms in post ischemia repair [42].

The main hub-gene of the PPI network, *AKT1*, codifies a member of the serine/threonine kinase family. The protein is a major constituent of the PI3K/Akt signaling pathway, activated in cell proliferation, survival, cell metabolism and angiogenesis. There are three members of the AKT family, AKT1, AKT2 and AKT3. AKT1 is the main isoform expressed in endothelial cells and can be activated in pro or anti-angiogenic pathways, according to the interacting molecules [43]. Based on this, we investigated, by in silico analysis, TFs potentially involved in the regulation of *AKT1* expression. Among regulatory factors identified we highlight the transcription factor *E2F1* (*E2F Transcription Factor 1*). The E2F family plays a fundamental role in regulating the cell cycle through balance of cell proliferation and apoptosis [44]. Therefore, E2F1 can act as a tumor suppressor or oncogene, through the PI3K/Akt signaling pathway [45, 46]. Studies have identified that activation of E2F1 stimulates the proliferative activity of AKT1, which in turn inhibits the apoptotic potential of E2F1 [47, 48]. Interestingly, *E2F1* (Log2FC = 3.17; FDR = 1.78E − 07) as well as *AKT1* (Log2FC = 2.57; FDR = 2.66E − 08) were shown to

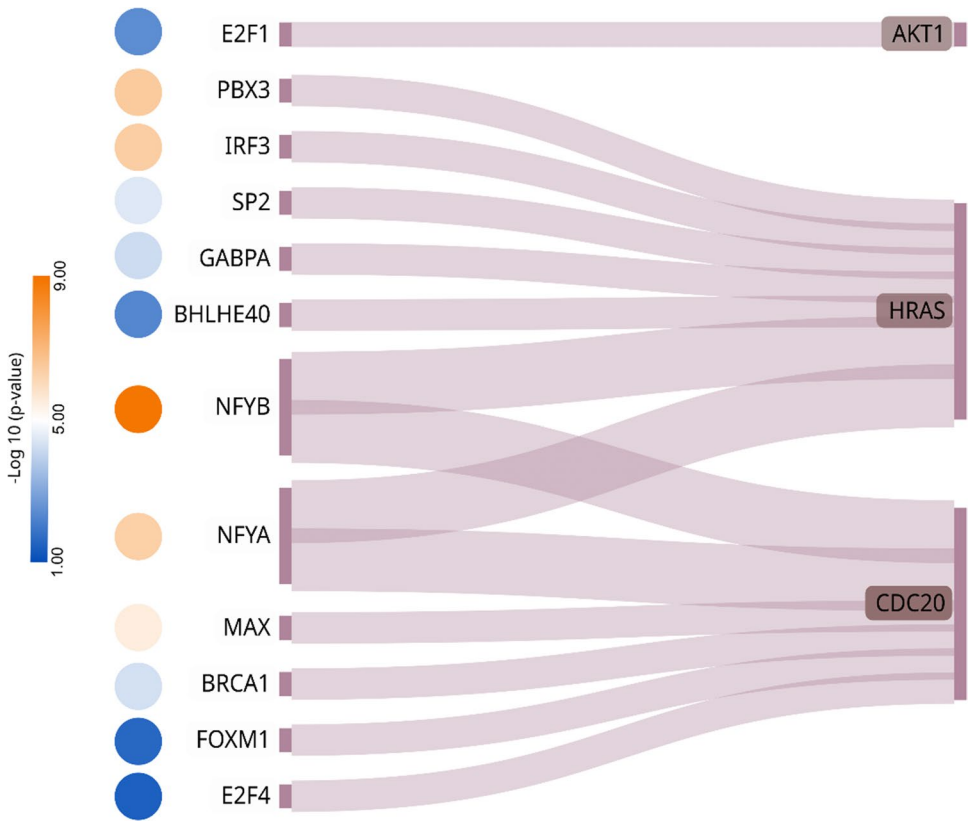

Fig. 7 Alluvial plot of transcription factors and correspondent hub-genes on the right of the in silico prediction of each TF

be upregulated in RNA-Seq analysis when stroke and non-stroke groups are compared.

Another target of *E2F1* is the *CDCA5* (*Cell division cycle associated 5*) or *soronin* gene, upregulated in the present analysis (Log2FC = 2.22; FDR = 9.48E − 05). *CDCA5* regulates the aggregation of sister chromatids during cell division [49], and it is upregulated in multiple cancers [50–52]. In vitro and in vivo investigations revealed that CDCA5 silencing suppresses cancer cell proliferation and tumorigenesis. It was shown that CDCA5, transcribed by E2F1, promotes growth of liver cancer cells by enhancing cell proliferation and inhibiting apoptosis via the AKT pathway [53].

Moreover, the AKT1 protein is activated by angiogenic stimuli and acts in endothelial cell mobilization following an ischemic damage [43]. Several growth factors can trigger its activation. Among them, we highlight the *EGFL7* gene (*EGF Like Domain Multiple 7*) also upregulated in RNA-Seq (Log2FC = 5.27; FDR = 5.8E − 23) in the present study. *EGFL7* gene codifies a secreted angiogenic protein, mainly expressed by endothelial cells in vasculogenesis during embryonic period [54, 55]. The relation between EGFL7 and the PI3K/Akt pathway has been observed in endothelial regeneration subsequent to vascular damage, with enhanced cell proliferation and invasion [56–58] and contributing to evidence that such interaction is also associated with physiological and pathological angiogenesis [54, 59, 60].

Therefore, AKT1 may interact with *E2F1*, *CDCA5* and *EGFL7*, with a central role in a variety of signaling cascades in endothelial cells. Thus, taken together, these data indicate that these genes upregulated in IS patients are most likely involved in the activation of important pathways associated with the post-stroke angiogenic processes.

The second hub-gene of PPI network, *HRAS*, is a GTPase of the Ras oncogene family. The protein is mainly involved in signal transduction, acting as a regulator of cell migration. Upregulation of the *HRAS* gene has been associated with cell proliferation, migration, invasion and angiogenesis in cancer [61]. Besides the important role of the GTPase in neoplasias, *HRAS* has also been associated with tissue repair signaling post-ischemia through hyperactivation of the PI3K/Akt pathway and alterations of Transforming Growth Factor β (TGF β) signaling [62].

The activation of immune response and the release of pro-inflammatory cytokines are well established mechanisms in stroke pathophysiology. However, after the ischemic event, there is a rise in the production of anti-inflammatory cytokines (including TGFβ), a requirement for brain recovery and repair[63]. *TGFB1*, upregulated in the analysis (Log2FC = 2.08; FDR = 1.02E − 07), is a cytokine related to development and tissue homeostasis. The signaling pathways of this group of growth factors has been related to vascular alterations, fibrosis and cancer. TGFβ acts in the resolution stage of angiogenesis, through endothelial cell differentiation and maintenance of vascular wall integrity [64].

The upregulation of *HRAS* was observed in a transcriptomic analysis of rats with permanent middle cerebral artery occlusion (MCAO) compared to the group with transient MCAO. The authors suggest that this profile may be related to the release of anti-inflammatory and neurotrophic factors for tissue recovery, in opposition to the inflammatory profile of the group with ischemia/reperfusion injury [65]. Interestingly, in the GO analysis in our study, *HRAS* was present in terms of "defense response", "immune response" and "chemotaxis".

Additionally, among the TFs obtained in the in silico prediction, *IRF3*, a potential TF of *HRAS*, is also upregulated in RNA-Seq. This factor activates the transcription of Interferons alpha and beta, as well as other Interferon-induced genes, with consequent activation of immunomodulatory pathways [66]. In a previous study using microglia cell culture, overexpression of *IRF3* was associated with upregulation of key anti-inflammatory cytokines and downregulation of pro-inflammatory cytokines. In addition, this IRF3-mediated anti-inflammatory profile was related to a higher Akt activation, suggesting the involvement of the PI3K/Akt signaling in this response [67]. Thus, the upregulation of *HRAS*, *IRF3* and *TGFB1* in IS patients is in accordance with previous findings, suggesting that in addition to the angiogenic stimuli, pathways of immunomodulation, inflammation resolution and tissue recovery may be activated in ECFCs.

The only downregulated hub-gene, *PIK3R1*, can codify three distinct proteins (p85α, p55α and p50α), regulatory subunits of Phosphoinositide 3-Kinase (PI3K), inhibiting the activity of the catalytic portion. Hence, the protein is involved in migration, proliferation and metabolism pathways [68]. The *PIK3R1* gene is present in "cell communication" and "cell surface receptor signaling pathway" gene ontology terms. The protein is expressed in various cellular types, in physiological and pathological conditions. *PIK3R1* suppression can lead to tumor progression, mediated by the catalytic portion of PI3K [69]. Therefore, this pattern of regulation can be associated with ECFCs proliferation.

This work has some limitations, such as the lack of mRNA and protein quantification and the small number of patients in each group. Additionally, the variety of clinical manifestations related to SCA can hamper the evaluation of IS separately. However, PCA and heatmap shows a distinct clustering between the groups with and without the complication, with a distinct gene expression profile. Regarding the treatment, all IS patients and one control individual were on transfusion therapy. Regular blood transfusions reduce endothelial activation and inflammation, thus modulating SCA phenotype. However, we highlight that in those patients, blood samples were collected

right before treatment. Furthermore, since cells from passages 3 to 5 were used for RNA extraction and sequencing, the transiently acquired phenotypes due to transfusion may have been reduced, not influencing the level of gene expression. In a previous validation study Milbauer et al. [70] demonstrated changes in ECFCs gene expression profile as consequence of cell culture stimulation with inflammatory cytokines. However, the gene expression returned to baseline after one expansion. These results contribute to our study model, indicating that the degree of expansion used occurred within a broad and safe window in which the acquired endothelial phenotypes would have disappeared and the gene expression of ECFCs reflects only the patient's genetic profile.

The evaluation of cultured ECFCs is another restraint of the present study, since these cells do not reside naturally in the brain. Nevertheless, there is increasing evidence that circulating endothelial cells are recruited to cerebral ischemic areas and can differentiate into mature cells and contribute to angiogenesis, tissue repair and regeneration in the ischemic brain [71–73].

Despite the severity of IS in SCA, widely accepted molecular targets are still lacking, especially in stroke outcome. The recovery is characterized by the combination of restorative and adaptive processes, with active participation of endothelial cells. Angiogenesis, the main mechanism highlighted in the present work, plays a central role in the regulation of ischemic diseases, and in the ischemic brain it has been shown to be correlated with survival and stroke recovery. In fact, angiogenesis has been proposed as a promising therapeutic and prognostic target. However, pathophysiological risk factors need to be considered. The responses and regulatory mechanisms underlying angiogenesis after injury may be more complex and not yet completely known.

Our previous sickle cell stroke study has investigated the differential expression of genes involved in endothelial cell biology through microarray technology [17]. Our preliminary observation revealed differentially expressed genes related to angiogenesis, inflammatory response, apoptosis, cell adhesion, coagulation. Now, to broaden our study, we performed transcriptomic analysis of the same samples except for two controls. RNA-Seq, a high throughput sequencing technology, is a valuable approach to identify gene expression alterations. As expected, our results were consistent between the two studies. In addition, the evaluation of the whole set of mRNA transcripts provided a more comprehensive view of EPCs molecular patterns in the context, pointing out to other genes directly relevant to endothelial cell biology, such as cell proliferation, migration and defense response, not previously identified.

Finally, we conclude that the comparative analysis of the gene expression profile of ECFCs from patients with versus without IS seems to indicate that in patients with stroke there is a persistent angiogenic process even after a long time this complication has occurred.

This is an original study, which may lead to new insights into the molecular basis of SCA ischemic stroke, contribute to a better understanding of the role of endothelial cells in stroke recovery and support further studies that might result in therapeutic strategies for this severe complication.

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Author contribution All authors participated in the design, interpretation of the study and review of the manuscript. Sample collection was performed by Mirta Tomie Ito and Roberta Casagrande Saez; neurological evaluation was conducted by Fernando Cendes; cell isolation and culture were performed by Sueli Matilde da Silva Costa, Mirta Tomie Ito, Roberta Casagrande Saez, Dulcinéia Martins de Albuquerque and Carolina Lanaro; review of medical records was done by Júlia Nicoliello Pereira de Castro, Sueli Matilde da Silva Costa, Victor de Haidar e Bertozzo, Mirta Tomie Ito and Thiago Adalton Rosa Rodrigues; transcriptomic data analysis and interpretation were performed by Júlia Nicoliello Pereira de Castro, Ana Carolina Lima Camargo, Bruno Batista de Souza, Sueli Matilde da Silva Costa and Mônica Barbosa de Melo; gene ontology analysis was conducted by Júlia Nicoliello Pereira de Castro, Sueli Matilde da Silva Costa, Ana Carolina Lima Camargo and Victor de Haidar e Bertozzo; protein–protein interaction network was constructed by Júlia Nicoliello Pereira de Castro and Thiago Adalton Rosa Rodrigues; In silico prediction of regulatory factors was conducted by Júlia Nicoliello Pereira de Castro and Ana Carolina Lima Camargo. The whole work was supervised by Sueli Matilde da Silva Costa, Fernando Ferreira Costa and Mônica Barbosa de Melo. The manuscript was written by Júlia Nicoliello Pereira de Castro, Sueli Matilde da Silva Costa and Mônica Barbosa de Melo. Júlia Nicoliello Pereira de Castro and Sueli Matilde da Silva Costa are equal contributors to the work.

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Declarations

Ethics approval This study was approved by the Research Ethics Committees of the Faculty of Medical Sciences, UNICAMP, in accordance with national guidelines (protocol nº 4.859.604) and with the Helsinki Declaration of 1975, as revised in 2008.

Consent to participate Informed consent was obtained from all individual participants included in the study.

Competing interests The authors declare no competing interests.

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