Integrated aqueous humor ceRNA and miRNA–TF–mRNA network analysis reveals potential molecular mechanisms governing primary open-angle glaucoma pathogenesis

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Purpose: To conduct an integrated bioinformatics analysis of extant aqueous humor (AH) gene expression datasets in order to identify key genes and the regulatory mechanism governing primary open-angle glaucoma (POAG) progression. Methods: Two datasets (GSE101727 and GSE105269) were downloaded from the Gene Expression Omnibus, and the messenger RNAs (mRNAs), microRNAs (miRNAs), and long noncoding RNAs (lncRNAs) were identified between controls and POAG patients. Differentially expressed (DE) mRNAs and DElncRNAs were then subjected to pathway enrichment analyses, after which a protein–protein interaction (PPI) network was generated. This network was then expanded to establish lncRNA–miRNA–mRNA and miRNA–transcription factor (TF)–mRNA networks. Results: The GSE101727 dataset was used to identify 2746 DElncRNAs and 2208 DEMRNAs, while the GSE105269 dataset was used to identify 45 DEMRNAs. We ultimately constructed a competing endogenous RNA (ceRNA) network incorporating 47 lncRNAs, six miRNAs, and 17 mRNAs. The proteins encoded by these 17 hub mRNAs were found to be significantly enriched for activities that may be linked to POAG pathogenesis. In addition, we generated a miRNA–TF–mRNA regulatory network containing two miRNAs (miR-135a-5p and miR-139-5p), five TFs (TGIF2, TCF3, FOS, and so on), and five mRNAs (SHISA7, ST6GAL2, TXNIP, and so on). Conclusion: The SHISA7, ST6GAL2, TXNIP, FOS, and DCBLD2 genes may be viable therapeutic targets for the prevention or treatment of POAG and are regulated by the TFs (TGIF2, HNF1A, TCF3, and FOS).

Key words: Differentially expressed genes, integrated analysis, primary open-angle glaucoma, transcription factors

Primary open-angle glaucoma (POAG) can result in increased intraocular pressure (IOP), irreversible damage to the optic nerve, and eventually blindness, and it is the leading cause of such blindness globally.[1] As of 2013, an estimated 64.3 million people between the ages of 40 and 80 were estimated to be affected by glaucoma worldwide, with this number being forecast to rise to 76.0 million and 111.8 million in 2020 and 2040, respectively.[2] Elevated IOP is known to be a major risk factor for POAG development that is normally regulated by homeostatic aqueous humor (AH) production and outflow.[3] Hundreds of different genes are believed to govern POAG development and progression. Indeed, as this disease develops through complex and poorly understood biological mechanisms, few effective biomarkers of POAG have been identified to date. While a recent study has explored the role of specific genes or transcription factors (TFs) in the context of POAG incidence, the full complexity of the interactions between long noncoding RNAs (lncRNAs), messenger RNAs (mRNAs), microRNAs (miRNAs), and TFs in this disease context remains to be fully elucidated.[4]

The recently proposed competing endogenous RNA (ceRNA) hypothesis suggests that specific RNAs can interact with one another through complementary miRNA response elements (MREs), enabling certain miRNAs to function as molecular “sponges” capable of sequestering and thereby modulating the functionality of specific target miRNAs.[5] Such a model enables researchers to develop putative ceRNA networks associating the expression of specific mRNAs with the activity of other noncoding RNA types including miRNAs, lncRNAs, and circular RNAs (circRNAs).[6] TFs and miRNAs serve as essential regulators of mRNA expression at the transcriptional and posttranscriptional stages, respectively, making them vital in both physiological and pathological contexts. These regulatory processes, however, do not happen in isolation, and as such, TFs and miRNAs have the potential to impact one another and to influence target mRNA expression in a complex and dynamic fashion that has yet

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to be studied in detail in the context of POAG development and progression.

Herein, we performed an integrative analysis of two POAG-related Gene Expression Omnibus (GEO) datasets in order to identify IncRNAs, miRNAs, and mRNAs that were differentially expressed (DE) in the AH of POAG patients relative to healthy controls. We then utilized the gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) databases to conduct functional enrichment analyses of identified DE genes (DEGs), after which a protein–protein interaction (PPI) network was constructed. We further conducted systematic integrated analyses of identified DElncRNAs, DEmiRNAs, and DEmRNAs based on their co-expression profiles in light of the ceRNA hypothesis, enabling us to conduct successive IncRNA–miRNA–mRNA and miRNA–TF–mRNA network analyses. The overall goal of these bioinformatic analyses was to identify genes related to POAG incidence and to provide novel insight into the molecular mechanisms governing this debilitating disease.

**Methods**

**Selection of datasets**

Two human AH microarray datasets were downloaded from GEO (http://www.ncbi.nlm.nih.gov/geo/) for use in this study, including GSE101727, which consists of IncRNA and miRNA expression profiles, and GSE105269, which comprises miRNA expression profiles. The GSE101727 dataset was based upon the GPL21827 platform (Agilent-079487 Arrayarray Human LncRNA microarray V4) and contained 10 control and 10 POAG samples,[7] while the GSE105269 dataset was based upon the GPL24158 platform (NanoString nCounter Human v3 miRNA Assay [NS_H_miR_v3a]) and included 11 control and 12 POAG samples.[8]

**Identification of DEGs**

Before DEG identification, dataset quality was evaluated using box plots and principal component analyses. The linear models for microarray data (LIMMA) and Affy R packages (http://www.r-project.org) were employed for differential expression analyses, with P value < 0.05 and \(|\log_2\) fold change (FC) \(\geq 1\) as the cutoff criteria for DEG identification. This strategy was used to identify DElncRNAs, DEmiRNAs, and DEmRNAs, after which they were arranged in heat maps and subjected to downstream analyses.

**Functional enrichment analyses**

The GO (http://www.geneontology.org) functions of identified DEGs, including enriched biological processes (BPs), molecular functions (MFs), and cellular components (CCs), were identified based upon available annotation to explore the functional roles of these proteins. Similarly, KEGG (http://www.genome.jp/kegg/) analyses were used to evaluate the pathways in which these DEGs are functionally enriched in the context of POAG. The R clusterProfiler package (http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html) was applied to conduct GO and KEGG analyses, with \(P \leq 0.05\) as the threshold of significance.

**ceRNA network construction**

Existing evidence has suggested that there were crosstalks among several types of RNA transcripts by MREs, including IncRNAs, miRNAs, and mRNAs. Accordingly, a previous study has mentioned that IncRNAs act as ceRNAs sponge to bind to miRNA through MREs, thereby regulating the expression level of mRNA.[5] Therefore, a ceRNA regulatory network was established to investigate the associations among DElncRNAs, DEmiRNAs, and DEmRNAs. Briefly, the miRcode (http://www.mircode.org/), a database including more than 10000 IncRNAs and providing putative miRNA target sites on the basis of comprehensive GENCODE (http://www.gencodegenes.org/) gene annotation, was employed to predict DElncRNAs, DEmiRNAs pairs. Meanwhile, the interactions between DEmiRNAs and DEmRNAs were assessed using miRanda (http://mirdb.org/index.html), miRMap (http://mirramp.mbc.nctu.edu.tw/), miRDB (http://mirdb.org/), TargetScan (http://www.targetscan.org/), and miRTarBase (http://mihtarbase.mbc.nctu.edu.tw/).[9] We merged the two relationship pairs of DElncRNAs–DEmiRNAs and DEmiRNAs–DEmRNAs that we obtained. Finally, we used Cytoscape software 3.4.0 (http://cytoscape.org/) to construct and visualize the ceRNA network.

**PPI network construction**

The Search Tool for the Retrieval of Interacting Genes (STRING; https://string-db.org/cgi/input.pl) database was employed for PPI network construction, with the resultant network being visualized using Cytoscape 3.4.0.

**miRNA–TF–mRNA regulatory network construction**

TF binding sites were initially predicted with the transcription factor-binding site (TFBS) tool R package, enabling us to predict TFs likely to regulate the transcription of 17 key POAG-related mRNAs present within our ceRNA network. Experimentally validated databases (miRDB, TargetScan, miRanda, miRMap, and miRTarBase) were then employed to predict miRNA–TF and miRNA–mRNA interactions, allowing us to construct a miRNA–TF–mRNA regulatory network, which was visualized using Cytoscape 3.4.0.

**Results**

**DE lncRNA, miRNA, and mRNA identification**

In the GSE101727 dataset, we identified 2746 DElncRNAs (1399 upregulated and 1347 downregulated) and 2208 DEmRNAs (1469 upregulated and 739 downregulated) on comparing POAG patient samples to control samples (\(P_{adj} < 0.05\) and \(|\text{FC}| \geq 2.0\)) (Supplementary File S1), among which the top IncRNAs with the most significant differences. These DElncRNAs and DEmRNAs were arranged in heat maps and volcano plots for ease of visualization [Fig. 1a].

Similar analyses of the GSE105269 dataset led us to identify 45 DEmiRNAs (23 upregulated and 22 downregulated) in POAG samples relative to normal control samples (\(P < 0.05\) and \(|\text{FC}| \geq 2.0\)) (Supplementary File S2), such as has-mir-451a, has-mir-3140-5p, has-mir-204-5p, has-mir-125b-5p, and others. These DEmiRNAs were additionally organized into volcano plots and heat maps [Fig. 1b].

**Functional enrichment analyses**

We next conducted GO and KEGG analyses to explore the potential functional roles of these identified DElncRNAs and DEmRNAs in the context of POAG. These DEGs were enriched in GO terms associated with nuclear-transcribed mRNA
catabolic processes, nonsense-mediated decay, translational initiation, cytosolic ribosomes, ribonucleoprotein complexes, and structural constituents of ribosomes [Fig. 2a–c]. Similarly, KEGG pathway analyses revealed these DEGs to be enriched in pathways associated with ribosomes, spliceosomes, and the proteasome [Fig. 2d].

Construction of a predictive ceRNA network
A putative IncRNA–miRNA–mRNA ceRNA network was next constructed by identifying IncRNAs and mRNAs targeted by miRNAs in the GSE101727 dataset. A total of 55 DEIncRNAs in this study were predicted to interact with eight miRNAs, while 604 mRNAs were predicted to be targets of six identified DEMiRNAs. By integrating these predictive analyses, we were able to generate a network of putative interactions among 47 DEIncRNAs, six DEMiRNAs, and 17 DEMRNAs [Fig. 3]. Among them, the differential expression of some genes reached more than six times. In addition, the genes in the ceRNA regulatory network were analyzed for function enrichment. From the results of KEGG function enrichment, it was found that the genes in the regulatory network were mainly enriched in transforming growth factor-β (TGF-β) signaling pathway, nuclear factor (NF)-kappa B signaling pathway, Wnt signaling pathway, ribosome, and others. Therefore, it demonstrates that the ceRNA relationship of the above genes may participate and play an important role in the occurrence and development of POAG through the above important signal pathways.

PPI network construction
A PPI network incorporating the 17 mRNAs identified in our ceRNA network was next constructed [Fig. 4]. This analysis revealed that subset of these proteins were predicted to interact with one another, suggesting that these key proteins may play interrelated roles in the context of POAG pathogenesis.

miRNA–TF–mRNA regulatory network construction
In our final analyses, we identified 383 putative TF-binding associations and six TFs predicted to regulate the expression of these 14 POAG-related hub RNAs. A predicted miRNA–TF–mRNA regulatory network incorporated two miRNAs (miR-135a-5p and miR-139-5p), five TFs (TGF-β-induced factor homeobox 2 [TGIF2], T-box transcription factor
5 [TBX5], hepatic nuclear factor 1α [HNF1α], transcription factor 3 [TCF3], and Fos proto-oncogene [FOS]), and five mRNAs (shisa family member 7 [SHISA7], ST6 β-galactoside α-2,6-sialyltransferase 2 [ST6GAL2], thioredoxin-interacting protein [TXNIP], FOS, and discoidin, CUB, and LCCL domain containing 2 [DCBLD2]) [Fig. 5]. We additionally determined that miR-135a-5p was predicted to inhibit two TFs (TGIF2 and TBX5) and three mRNAs (SHISA7, ST6GAL2, and TXNIP), while miR-139-5p was predicted to inhibit four TFs (TGIF2, HNF1A, TCF3, and FOS) and two mRNAs (FOS and DCBLD2). These findings suggested that different miRNAs were able to coregulate the expression of specific genes, indicating that these miRNAs may play complex and crosstalk roles in the context of POAG incidence. Of the five TFs identified in these analyses, TGIF2 was predicted to regulate the expression of four mRNAs (ST6GAL2, SHISA7, TXNIP, and DCBLD2). In addition, FOS was predicted to serve as a regulator of DCBLD2, which was also coregulated by TCF3 and HNF1A in our regulatory network. Similarly, DCBLD2 was coregulated by TGIF2, HNF1A, TCF3, and FOS.

Discussion
Elevated IOP can result in axonal compression of the optic nerve and the apoptotic death of retinal ganglion cells (RGCs), thereby impairing the normal homeostatic balance between AH production and outflow within the anterior chamber. Given the pathological nature of such dysregulation, molecular mechanisms regulating the AH and IOP can influence glaucoma development, and previous studies have identified multiple
pathways and individual genes associated with POAG incidence. \[11\] Few studies to date, however, have conducted systematic analyses to identify the relationships between IncRNAs, miRNAs, TF, and mRNAs in this pathological context. Herein, we, therefore, conducted a comprehensive integrative bioinformatics analysis of the molecular basis for POAG pathogenesis, leading us to identify multiple genes and TFs that are potentially associated with this condition. By analyzing two extant microarray datasets, we were able to identify 2746 DElncRNAs and 2208 DEmRNAs in the GSE101727 dataset and 45 DEMiRNAs in the GSE105269 dataset. Using GO and KEGG pathway analyses, we further explored the potential pathogenic roles of these IncRNAs and miRNAs and thereafter constructed IncRNA–miRNA–mRNA and miRNA–TF–mRNA networks to evaluate their interrelated regulatory functions.

In eukaryotes, both TFs and miRNAs serve as primary regulators of gene expression, with TFs serving to directly control DNA transcription\[12,13\] and miRNAs functioning by binding to conserved MREs to disrupt target mRNA translation at the posttranscriptional level.\[14,15\] In previous analyses, miRNAs have been shown to control anterior chamber shape, IOP, and RGC apoptosis via the regulation of specific target genes.\[16\] In addition, there is evidence that miRNAs can regulate diverse biochemical pathways in the context of glaucoma,\[17\] and miRNAs including miR-125b-5p have been detected at elevated levels in human AH relative to human serum.\[18\] At a functional level, miR-139 has been found to regulate the Wnt signaling pathway, which is a key mediator of TGF-β1–induced fibrosis.\[19\] As for TGF-β signaling, it served to regulate the AH environment by regulating ocular hypertensive mediator gene expression,\[20\] suggesting that this pathway may be associated with POAG incidence. We found that miR-125b-5p and mir-24-3p were downregulated in our ceRNA network, whereas miR-135a-5p, miR-193a-3p, miR-451a, and miR-139-5p were upregulated. Consistent with our result, Drewry et al.\[8\] reported that miR-125b-5p was significantly higher in POAG.\[8\] We observed that miR-125b-5p was able to target three glaucoma-associated genes, RNA-binding motif protein 7 (RBM7), ornithine decarboxylase antizyme 2 (OAZ2), casein kinase II subunit α (CSNK2α), although none of these has been previously reported in glaucoma. In addition, 11 IncRNAs served as the nodes finally in miR-125b-5p–regulated pathways, which were significantly higher in comparison to other IncRNAs. Furthermore, the GO and KEGG pathway analyses were employed to investigate enriched biological functions. The DEMiRNA in the IncRNA–miRNA–mRNA ceRNA network-related KEGG analysis indicated that TGF-β signaling pathway, NF-kappa B signaling pathway, Wnt signaling pathway, ribosome, and others could play an important role in the development of POAG. Fukushima et al.\[21\] found that the nuclear exosome targeting complex component RBM7 was highly upregulated in the fibrotic lung and plays fundamental roles in fibrosis development.\[21\] Therefore, we speculate that the IncRNA–miR-125b-5p–RBM7 network is likely to play a part through TGF-β signaling pathway in

Figure 3: The POAG IncRNA–miRNA–mRNA ceRNA interaction network. Triangles, rectangles, and ovals correspond to IncRNAs, miRNAs, and mRNAs, respectively, with blue and red corresponding to downregulation and upregulation, respectively. Gray edges indicate interactions between RNAs. ceRNA = competing endogenous RNA, DElncRNAs = differentially expressed long noncoding RNAs, DEMiRNAs = differentially expressed microRNAs, DEmRNAs = differentially expressed messenger RNAs, POAG = primary open-angle glaucoma
the development of POAG. Besides, both miR-135a-5p and miR-139-3p can suppress the expression of TGIF2, which is a transcriptional regulator that plays an essential role in cellular differentiation, proliferation, and embryonic development.\[^{22,23}\]

Herein, we identified TGIF2 as a direct miR-135a-5p and miR-139-5p target in the context of POAG, suggesting that the ability of these two miRNAs to modulate POAG progression may be attributed to their ability to control TGIF2 expression.

FOS (c-Fos) is an inducible TF and a member of the activator protein 1 (AP1) family.\[^{24}\] Through PPIs, FOS can additionally govern the transcriptional activity of other TFs.\[^{25,26}\] We found that FOS was incorporated into our ceRNA and PPI networks, suggesting that it may be a key regulator of POAG development and progression. FOS expression is closely associated with the apoptotic death of various neuronal cell types,\[^{27,28}\] and there is also evidence that it plays a role in mediating RGC damage,\[^{29}\] underscoring its potential relevance to the etiology of POAG. TXNIP, which was first detected in 1,25-dihydroxyvitamin D3-treated HL-60 cells,\[^{30}\] can increase cellular sensitivity to oxidative stress and can drive bioenergetic imbalance, autophagy, and apoptotic cell death.\[^{31,32}\] Oxidative stress is one of the primary drivers of IOP-associated RGC death, and TXNIP upregulation is observed in the context of RGC death induced by both optic nerve transection and elevated IOP.\[^{33,34}\] When the expression of TXNIP is decreased, this is sufficient to inhibit RGC death.\[^{35}\] RGC and optic nerve axonal degradation are the primary diverse causes of visual impairment and blindness in POAG patients. These data thus confirm that TXNIP is a key target in the context of POAG progression. Herein, we determined that miR-135a-5p and two TFs (TGIF2 and TBX5) were able to regulate TXNIP expression in the AH of POAG patients. DCBLD2 is a key regulator of cellular proliferation and vascular remodeling.\[^{36}\] Our miRNA–TF–mRNA network suggested that HNF1A, TCF3, FOS, and TGIF2 were able to stimulate DCBLD2 expression, indicating that DCBLD2 may influence POAG pathogenesis through these transcriptional mechanisms.

**Conclusion**

In summary, in the present study, we identified a novel subset of genes that are DE in the AH of POAG patients relative to the AH of healthy controls, suggesting that these genes may be closely linked to the development and progression of this debilitating condition. Through our comprehensive bioinformatics analyses, we highlighted novel transcription and posttranscriptional regulatory mechanisms governing POAG incidence. We further identified five genes (SHISA7, ST6GAL2, TXNIP, FOS, and DCBLD2) that may represent viable targets for the treatment or prevention of POAG and that are regulated by the TFs (TGIF2, HNF1A, TCF3, and FOS). While our findings provide new insight into the molecular etiology of POAG, further experimental validation of our results will be essential for confirming their relevance and exploring their therapeutic potential.

**Supplementary information**

Additional supporting information may be found in the online version of this article:

File S1 and S2. The top 20 differentially expressed lncRNAs (a) and mRNAs (b) in the AH on comparing POAG patient to control samples in the GSE101727 dataset
Chapter 11-MicroRNA target prediction in glaucoma. Prog Brain Res 2015;220:217-40.
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### Supplementary File S1: The top 20 differentially expressed lncRNAs (a) and mRNAs (b) in the AH on comparing POAG patient to control samples in the GSE101727 dataset

| Type       | Gene (IncRNA) | logFC     | P       |
|------------|---------------|-----------|---------|
| Upregulate | G030772       | 6.16267299| 4.88E-05|
|            | G089593       | 5.675029049| 1.15E-05|
|            | AK130076      | 5.594505028| 4.28E-07|
|            | his-1_RNA_dna | 5.520955873| 1.12E-06|
|            | G030931       | 5.354702271| 1.38E-05|
|            | uc. 420       | 5.340346004| 3.39E-05|
|            | AC007383.4    | 5.31385727 | 2.04E-06|
|            | G075678       | 5.242404881| 2.34E-05|
|            | G017745       | 5.200545058| 3.31E-05|
| Downregulate| AX747413      | 5.183745365| 2.49E-05|
|            | G087478       | -7.331093179| 3.47E-08|
|            | G012197       | -7.397532734| 1.17E-08|
|            | G062833       | -7.489679005| 1.28E-09|
|            | G066556       | -7.58628678 | 1.21E-13|
|            | RP11-567A7.2  | -7.892728347| 8.19E-12|
|            | G028246       | -8.256822949| 1.19E-05|
|            | G055948       | -8.435839215| 4.90E-06|
|            | AX747639      | -8.624651459| 2.86E-13|
|            | G024686       | -8.902864682| 1.57E-13|
|            | G040037       | -9.866278335| 9.70E-18|

| Type       | Gene (mRNA)  | logFC     | P       |
|------------|--------------|-----------|---------|
| Upregulate | MT-ATP6      | 6.921437354| 3.29E-05|
|            | RPL37        | 6.116301304| 1.02E-08|
|            | MT-ND4       | 5.57813416 | 0.001804851|
|            | FAM124A      | 5.510547269| 8.88E-07|
|            | ZBTB4        | 5.401294094| 7.34E-06|
|            | PRRT1        | 5.39613156 | 1.47E-05|
|            | NOL9         | 5.228657401| 2.26E-05|
|            | RBM7         | 5.178296974| 9.24E-07|
|            | RPS2P32      | 5.104749248| 1.89E-05|
|            | HGC6.3       | 5.050984366| 6.81E-06|
| Downregulate| PEL1         | -6.577541159| 4.84E-06|
|            | PPP1R16B     | -6.624537016| 9.91E-07|
|            | ORAI2        | -6.741647502| 2.10E-06|
|            | SEMA4B       | -6.766480122| 1.72E-09|
|            | FJCG1        | -6.829467762| 4.06E-07|
|            | ITGA5        | -6.965581785| 8.29E-05|
|            | IL22         | -7.187951208| 2.71E-10|
|            | OR10J1       | -7.427456955| 1.45E-05|
|            | MAGEA12      | -7.912221816| 7.41E-12|
|            | NIPAL3       | -8.520859862| 8.62E-11|

### Supplementary File S2: The top 20 differentially expressed miRNAs in the AH on comparing POAG patient samples to control samples in the GSE105269 dataset

| Type       | Gene (miRNA) | logFC     | P       |
|------------|--------------|-----------|---------|
| Upregulate | hsa-miR-451a | 1.099764108| 0.032177995|
|            | hsa-miR-3140‑5p| 0.7593001 | 0.034883538|
|            | hsa-miR-3190‑3p| 0.860045773| 0.017180336|
|            | hsa-miR-194‑5p| 0.857009272| 0.020429938|
|            | hsa-miR-135a‑5p| 0.642775355| 0.045576341|
|            | hsa-miR-320e | 0.59668708 | 0.024266619|
|            | hsa-miR-648 | 0.5066355 | 0.007511757|
|            | hsa-miR-137 | 0.497752609 | 0.049674124|
|            | hsa-miR-224‑5p| 0.497159513| 0.04279984|
|            | hsa-miR-1244 | 0.482940146 | 0.007688872|
| Downregulate| hsa-miR-484 | -0.505411455 | 0.034413007|
|            | hsa-miR-431‑5p| -0.50830537 | 0.018242336|
|            | hsa-miR-1910‑5p| -0.534734696| 0.032714754|
|            | hsa-miR-2682‑5p| -0.537553037| 0.006452898|
|            | hsa-miR-517c‑3p| -0.54289098 | 0.00659242|
|            | hsa-miR-375 | -0.545806863 | 0.004270409|
|            | hsa-miR-106b‑5p| -0.579127165| 0.035851945|
|            | hsa-miR-3195 | -0.599964555 | 0.031150973|
|            | hsa-miR-125b‑5p| -0.630026536| 0.009991458|
|            | hsa-miR-204‑5p| -0.844884632| 0.018554867|