The Role of hsa-miR-548l Dysregulation as a Putative Modifier Factor for Glaucoma-Associated *FOXC1* Mutations

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Abstract: Mutations of the *FOXC1* transcription factor are involved in a variety of autosomal dominant ocular anterior segment defects, ranging from Axenfeld-Rieger malformations to isolated glaucoma in some patients. In this study we have evaluated the possible role of the c.*734A>T *FOXC1* variant as a modifier factor of the activity of two *FOXC1* mutations previously identified in families primarily affected by dominant glaucoma (haplotypes p.G447_G448insDG-c.*734A>T and p.I126S-c.*734A>T). Previous bioinformatic analyses indicated that the c.*734A>T variant is located in a potential target sequence for hsa-miR-548l. Co-expression of this miRNA with a reporter cDNA construct in which the wild-type 3'UTR sequence of *FOXC1* was fused to the 3'-end of the firefly luciferase coding region, led to approximately 20% decreased luciferase activity compared to the controls, confirming the presence of a target sequence for hsa-miR-548l. In contrast, this miRNA did not show any effect on the luciferase activity associated with the mutant 3'UTR *FOXC1* sequence, showing that it resulted in a loss-of-function of the has-miR-548l target sequence. In addition, functional evaluation of the two glaucoma-associated haplotypes revealed increased protein levels and transactivation, compared to the corresponding individual coding mutations (approximately 1.2-fold on average). These data support the role of hsa-miR-548l as a regulator of *FOXC1* translation and provide evidence for the c.*734A>T variant as a modifier factor for the activity of coding glaucoma-associated *FOXC1* mutations.

Keywords: congenital glaucoma, dominant glaucoma, *FOXC1*, glaucoma, miRNA-548l, modifier factor.

1. INTRODUCTION

Glaucoma is a highly heterogeneous, irreversible and progressive optic neuropathy produced by the death of the retinal ganglion cells, which results in the degeneration of the optic nerve and eventually in blindness. Raised intraocular pressure (IOP) is the primary risk factor for developing glaucoma. It has been proposed that oxidative stress plays a primary role among the multiple factors involved in glaucoma pathogenesis, affecting both the cells of the trabecular meshwork and the ganglion cells of the retina [1,2]. Most glaucoma cases behave as a late-onset complex disease and in a minority of patients the disease shows an early-onset and follows Mendelian inheritance. The existence of incomplete penetrance and variable expressivity in some Mendelian glaucoma cases indicate the influence of unidentified modifier factors (genetic, stochastic and/or environmental) on the final phenotypic outcome [3,4].

The *FOXC1* protein is a member of the FOX class of transcription factors, which regulate, craniofacial, ocular and cardiovascular development [5]. Mutations in the Axenfeld-Rieger *FOXC1* gene are involved in different types

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of autosomal dominant anterior eye segment defects, including syndrome type 3 (ARS; MIM#602482). Increased risk for glaucoma and variable iris or extra-ocular abnormalities are commonly associated with mutations in this gene [6]. Although most *FOXC1* mutations reduce the *FOXC1* transactivation ability [7], we have recently described hypermorphic variations associated with dominant glaucoma (Escribano, personal communication). The regulation of *FOXC1* gene expression is poorly understood, particularly at the level of translation. Recently, several functional cis-regulatory elements located in the UTRs of this gene have been identified, including a terminal oligopyrimidine tract (Escribano, personal communication), which regulates translation initiation, and target sequences for some miRNAs [8-10]. Disruption of these regulatory sequences alter *FOXC1* translation and, therefore, they have the potential to be pathogenic.

It is well documented the role of MicroRNAs (miRNAs) as key regulators of gene expression at the post-transcriptional level in different normal and pathological

biological processes [11,12]. However, the study of this type of gene regulators in ocular physiology and pathology is very limited. In that sense, it has been shown, for example, that miR-200c regulates trabecular meshwork contraction and intraocular pressure [13] and that miR-204 target genes are involved in apoptosis, reticulum stress and inflammation in human trabecular meshwork cells [14]. In addition, we have reported that the *16T>C *WDR36* mutation, identified in one POAG patient, may form part of a predicted target sequence for miR-9 and miR-224 [15].

In this study, we show that *FOXC1* mRNA translation is regulated by hsa-miR548-l and that disruption of its target sequence by the c.*734A>T *FOXC1* mutation modifies the activity of two coding mutations of the same gene.

2. MATERIALS AND METHODS

To evaluate the effect of the c.*734A>T *FOXC1* variant as a modifier factor, two haplotypes (p.G447_G448insDG-c.*734A>T and p.I126S-c.*734A>T) identified in families affected by dominant glaucoma were cloned and functionally evaluated by miRNA target reporter and transactivation assays. Informed consent was obtained from the patients included in this study. The informed consent was approved by the Ethics Committee for Human Research of the Hospital Clínico San Carlos, Madrid (Spain), and followed the tenets of the Declaration of Helsinki.

2.1. Cloning of *FOXC1* and site directed mutagenesis

The wild-type *FOXC1* coding sequence, obtained from a commercial cDNA (Origene, NM_001453), was subcloned into the *EcoRI* and *BamHI* restriction sites of the modified mammalian cell expression vector pcDNA3.1(-) (C-terminal Myc-Tagged) [3] by directional PCR using the following primers: 5'GGGAATTCGGGGCCATGCAGGCGCGCTACT3' (P1) and 5'GTGGATCCCCAACTTGCTACAGTCGTAGA3' (P2) (*EcoRI* and *BamHI* sequences are indicated in bold, respectively). This cDNA construct was used as a template for the site-directed mutagenesis of the p.I126S mutation using the QuickChange Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer's instructions, and the following primer set: 5'GCTGGCAGAACAGCAGCCGCCACAACCTCTCG3' (forward) and 5'CGAGAGGTTGTGGCGGCTGCTGTTCTGCCAGC3' (reverse). The p.G447_G448insDG variant was directly amplified from the genomic DNA of one carrier diagnosed with adult-onset dominant glaucoma (I:2, Fig. 1B) by directional PCR using the primers P1 and P2. The obtained amplicon was cloned into the *EcoRI/BamHI* restriction sites of the modified vector pcDNA3.1(-). A commercial cDNA, containing 1.8Kb of the wild-type 3'-UTR *FOXC1* sequence (Origene, SC216457), was used as a template for the site-directed mutagenesis of the c.*734A>T and c.*736A>T mutations using the QuickChange Site-directed Mutagenesis Kit, and the primers and PCR conditions indicated in Table 1. The mutagenesis products were sequenced to confirm the

presence of the mutation and were subcloned into the *EcoRI* and *NotI* restriction sites of the pMirTarget vector (Origene) to avoid undesirable mutations in the vector. In order to obtain the mutant haplotypes p.I126S-c.*734A>T and p.H446_G447insGD-c.*734A>T, the cloned mutant 3'-UTR sequence was amplified using the following primers, which contained the *HindIII* restriction site indicated in bold: 5'GCAAGAAGCTTCACACCCTCAAAGCCG3' (forward) and 5'CTCAAGCTTGGAGCGGCAGATAGCAGG3' (reverse). The PCR product was fused to each mutant coding region, previously cloned into the pcDNA3.1(-) plasmid, using the *HindIII* restriction site. The *FOXC1* wild-type coding sequence and 3'-UTR were also fused following a similar approach.

2.2. miRNA target reporter assays

The putative miRNA target sequences in the region surrounding the *FOXC1* c.*734A>T mutation, were identified using the *MicroSniPer* software (<http://cbdb.nih.gov/microsniper>) [16]. To analyze the possible effect of this variant, the 1.8-Kb *FOXC1* 3'-UTR sequence (Origene, SC216457) was fused to the 3'-end of the firefly luciferase coding region (cloned into the pMirTarget vector; OriGene, PS100062), using the *SgfI/MluI* restriction sites. This cDNA was used as a template to obtain the variants by site-directed mutagenesis, as described above. The miRNA target reporter assays were performed in triplicate in 24-well plates. The HEK-293T cells were transfected with 250 ng of each 3'-UTR *FOXC1* target construct (wild-type or mutant) and 500 ng of the recombinant pCMV-MIR vector encoding either hsa-miR-548l (SC400522, Origene) or hsa-miR-548h (SC400512, Origene) using the Superfect Transfection Reagent according to the manufacturer's protocol. After a 48h incubation, the luciferase activity was measured using the Luciferase Assay System (Promega) according to the manufacturer's instructions. RFP and LDH were detected via western blot as the transfection and loading controls, respectively, quantitated by densitometry ($n \geq 3$), and significant differences were analyzed using the *t*-test.

2.3. Transactivation assays

The *FOXC1* transactivation assays were performed using the luciferase assay previously indicated. A 600-bp fragment of the human *CXCR4* gene promoter, which contains one FOX-binding element (FBE)¹⁷, was cloned into the *NheI/NcoI* restriction sites of the pGL3-basic vector (Promega) via directional PCR using normal human genomic DNA as a template and the following primers: 5'TCTGGCTAGCGCGCGGGGAATGGCGTTGG3' and 5'TCCATGGTAACCGCTGGTTCTCCAG3' (the *NheI* and *NcoI* sequences are indicated with bold letters, respectively). HEK-293T cells in 24-well tissue culture plates (2.5×10^5 cells/well) were transfected with 500 ng of the *FOXC1* expression vector, along with 50 ng of the recombinant pGL3-basic-*CXCR4* luciferase reporter, and 50 ng of the pMirTarget vector (Origene), which expresses red fluorescent protein (RFP) as a transfection control. The total

amount of transfected DNA was equalized using the empty vector. The transactivation assays were performed 48 h after the transfection. The cells were harvested and assayed for firefly luciferase activity as previously indicated. FOXC1, RFP and LDH were detected by Western blot as the expression, transfection and loading controls, respectively. The RFP and LDH bands were quantitated via densitometry ($n \geq 3$) and significant differences were analyzed using the *t*-test.

2.4. Statistical analyses

The statistical comparisons between the groups were performed using either the *t*-test or the one-way analysis of variance (ANOVA). The data were statistically processed using the SigmaStat 2.0 software (SPSS Science).

3. RESULTS

3.1. Identification of the c.*734A>T FOXC1 variant in dominant glaucoma

In a previous genetic study on dominant glaucoma we identified two probands who carried the *FOXC1* mutations p.I126S and p.G447_G448insDG along with the non-coding mutation c.*734A>T in the same gene (Escribano, personal communication). The three variants were present in the heterozygous state and the segregation analysis showed that the two coding mutations were coupled with the c.*734A>T mutation, forming two rare haplotypes (p.I126S—c.*734A>T and p.G447_G448insDG—c.*734A>T) (Fig. 1). The 3'-UTR variant was absent in 348 control subjects (696 chromosomes). The first haplotype is associated with remarkable phenotypic variability, ranging from isolated congenital and juvenile glaucoma to glaucoma with Axenfeld-Rieger anomaly, whilst the second haplotype is present in primary open-angle glaucoma cases diagnosed in their fifties or sixties (Escribano, personal communication).

3.2. miRNA assay

Previous bioinformatic analysis of the *FOXC1* 3'-UTR sequence with the *MicroSNiPer* software showed that the variation c.*734A>T localized to a putative seed target sequence for hsa-miR-548l (Fig. 2A). This nucleotide sequence was highly conserved in the four analysed mammals (Fig. 2B). To assess whether the c.*734A>T mutation is in fact embedded in the target sequence for this miRNA, the wild-type 3'-UTR and two 3'-UTR versions containing the c.*734A>T or c.*736A>T (control) mutations, were coupled to the 3' end of the luciferase coding region. A transient co-expression of the wild-type 3'-UTR construct with cDNAs encoding either hsa-miR-548l or hsa-miR-548h (negative miRNA control) was followed by an *in vitro* luciferase activity assay. hsa-miR-548h was used as a negative control because its target sequence was inferred to map near the mutation site (Fig. 2A). The results showed that the first miRNA reduced the wild-type 3'-UTR-associated luciferase activity to 80% of the control assay (Fig. 3, wild-type). In contrast, and as expected, the control

miRNA had no significant effect on luciferase activity (Fig. 3, wild-type). The two mutant 3'-UTR sequences did not respond to the miRNAs used in the assays (Fig. 3, c.*734A>T and c.*736A>T). These results support the presence of a functional hsa-miR-548l target sequence in the 3'-UTR of *FOXC1* and shows that its disruption by c.*734A>T specifically eliminates the effect of this miRNA on luciferase activity, showing that it is a loss-of-function mutation. Western blot analyses of RFP and LDH showed no significant differences in the transfection efficiency or sample loading, respectively (Fig. 3).

3.3. Transcriptional activity

To evaluate the effect of the c.*734A>T variant on *FOXC1* transactivation, the mutant 3'-UTR sequence was fused to the wild-type *FOXC1* coding sequence. In addition, cDNA constructs encoding the two *FOXC1* haplotypes identified in glaucoma patients were also obtained. Each of the individual cDNA constructs was transiently co-transfected with the luciferase reporter gene coupled to the *CXCR4* promoter region, which contains one *FOXC1* binding element. In addition, *FOXC1* levels were estimated by western blot and quantitated by densitometry. According to the luciferase results described in the previous section, the variant c.*734A>T, coupled to the wild-type *FOXC1* coding sequence exhibited approximately 1.3-fold increased transactivation, compared to the wild-type construct (Fig. 4A). As expected, the cDNA constructs encoding either the previously known hypermorphic p.G447_G448insDG or hypomorphic p.I126S (Escribano, personal communication), variants, coupled to the wild-type 3'UTR sequence showed, respectively, a 150% increased or 20% decreased transactivation compared to the control (Fig. 4A). The two pathogenic haplotypes (p.G447_G448insDG—c.*734A>T and p.I126S—c.*734A>T) showed higher transactivation than the corresponding isolated coding mutation coupled to the wild-type 3'UTR sequence (approximately 1.2- to 1.3-fold (Escribano, personal communication) on average; Fig. 4A). The *FOXC1* protein levels associated with each of these cDNA constructs correlated with *FOXC1* transactivation values (Fig. 4B), further supporting that c.*734A>T disrupts the hsa-miR548l target sequence leading to improved mRNA stability and thus, to increased protein and transactivation levels. Western blot analyses of RFP and LDH were also used as controls for the transfection efficiency and sample loading, respectively, and revealed no significant differences.

4. DISCUSSION

In previous studies we identified the c.*734A>T (rs35717904) mutation in two families affected by dominant glaucoma and in one patient diagnosed with non-dominant unilateral late-onset congenital glaucoma at the age of 5 years (Escribano, personal communication). In this study we have evaluated the role of this variant on both *FOXC1* activity and glaucoma development. c.*734A>T has been described at low frequencies in Europeans (Ensembl variation database) and was not found in 696 control chromosomes, showing that it is an infrequent variant.

Bioinformatics analyses of the c.*734A>T mutation led to the identification of hsa-miR548l as a putative miRNA involved in regulating *FOXC1* translation (Escribano, personal communication). Our functional analyses confirmed that disruption of the hsa-miR548l target sequence by the c.*734A>T mutation results in a loss-of-function of this regulatory sequence, leading to improved mRNA stability and therefore, to moderately increased protein and transactivation levels. The sequence of the hsa-miR548-l target was present in the four mammalian *FOXC1* genes analysed, indicating a relatively recent evolutionary origin and that this miRNA may regulate *FOXC1* gene expression in these species. Currently, more than 120 members of the hsa-miR548 gene family are recognized (miRBase, release 21; <http://www.mirbase.org/>), which regulate to more than 3500 putative target genes [18]. This gene family is primate specific and is derived from *Made1* transposable elements, and cancer-related regulatory roles have been suggested for hsa-miR-548 [18]. Our results also support that one member of this family, mir-584l, participates in *FOXC1* translation regulation, contributing to understand the mechanisms involved in *FOXC1* expression regulation at the posttranscriptional level. It is known that *FOXC1* expression is regulated by miR-204, which binds to two target sequences in the 3'-UTR and it has been proposed a crucial role for miR-204-*FOXC1* interaction in endometrial cancer progression [8]. Moreover, our study supports that dysregulation of mir-584l plays a role in glaucoma as a modifier factor for the activity of other two *FOXC1* mutations, being one of only a few miRNAs known to be related with this disease. Dysregulation of miRNAs and their targets has the potential to be pathogenic, not only as disease causing genes but also as modifier factors [19]. In this line, the two pathogenic haplotypes identified in this study, which include the c.*734A>T variant (p.I126S—c.*734A>T and p.G447_G448insDG—c.*734A>T), showed higher transactivation than the individual coding mutations. Therefore, the loss-of-function associated with p.I126S was partially balanced by the presence of c.*734A>T, leading to improved mRNA stability, increased protein levels and higher transactivation than the single p.I126S mutation. This increased activity might explain why haplotype p.I126S—c.*734A>T is associated with glaucoma, whilst the single related mutation p.I126M, whose reduced activity is similar to that of individual p.I126S (Escribano, personal communication), has been found associated with ARS and glaucoma [6]. p.I126M has also been proposed to be a positive control mutant for activity studies in the forkhead family of transcription factors [7]. Decreased transactivation of both p.I126S and p.I126M mutations has been attributed to impaired DNA binding affinity, probably due to structural alterations in the forkhead domain alpha-helix 3, which is predicted to play a role in major DNA groove recognition. Although protein phosphorylation and subcellular localization differences between these two mutations have been reported, these functional alterations do not correlate with the severity of the associated phenotypes. Therefore, the modifier effect of c.*734A>T on p.I126S and

p.G447_G448insDG *FOXC1* mutations may contribute to the final phenotypic difference. Regarding pathogenicity of p.G447_G448insDG, it has been proposed that its increased transcription activity may depend on a gain-of-function that results in altered binding to transcription factors (Escribano, personal communication). Sequence analysis of the *FOXC1* 3'-UTR in glaucoma patients carrying coding *FOXC1* mutations may contribute to confirm the role of c.*734A>T as a modifier factor in this disease.

FOXC1 mutations are known to be associated with remarkable phenotypic variability [20,21], which suggests an important role for modifier factors (genetic, environmental and/or stochastic) on the phenotypic outcomes. According to the previously formulated hypothesis of critical activity thresholds for *FOXC1* and the related protein PITX2 [22], we have proposed that the phenotypic variability associated with *FOXC1* mutations may depend on narrow intervals of residual transactivation related to the individual *FOXC1* genotypes (Escribano, personal communication). In this conceptual framework, the modifier effect of the c.*734A>T variant may contribute to the overall genotypic-associated transactivation that influence the final phenotypic outcome.

5. CONCLUSION

This study shows that hsa-miR548-l regulates *FOXC1* translation, contributing to better understand the fine regulation of the biological function of this transcription factor. Our data also support that the c.*734A>T *FOXC1* mutation disrupts the seed target sequence of this miRNA, resulting in a loss-of function and acting as a modifier factor for the activity of other *FOXC1* mutations. This modifier effect may help to explain the phenotypic variability observed in *FOXC1*-associated glaucoma. Finally, the identification of new miRNAs as regulators of *FOXC1* translation provides new potential therapeutic targets to correct altered *FOXC1* levels, which are present in diseases ranging from Axenfeld-Rieger malformations to isolated dominant glaucoma.

6. CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

7. ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. (1). *FOXC1* haplotype segregation in two families affected by autosomal dominant glaucoma. Pedigrees of families PCG73 (A) and 556-10 (B). The variants in the two vertical lines represent the haplotypes. The black, dark grey and light grey symbols indicate congenital glaucoma, juvenile glaucoma and adult-onset primary open-angle glaucoma, respectively. +: wild-type allele. insDG: p.G447_G448insDG. The complete pedigrees have previously been reported (Escribano, personal communication).

Fig. (2). (A) The c.*734A>T *FOXC1* mutation disrupts a putative target sequence for hsa-miR-548l. The target miRNAs were predicted with the MicroSNiPer software. The c.*734A>T mutation maps at the predicted target sequence for hsa-miR-548l. The arrow indicates the position of the mutant nucleotide. The predicted binding site of the control hsa-miR548h is also indicated. Note that the c.*734A>T mutation does not affect the interaction of this control miRNA with its target site. The asterisk indicates the position of the c.*736A>T mutation, a control of the seed sequence. Vertical lines and dots in the predicted miRNA target sequence indicate perfect Watson-Crick and G:U wobble pairings, respectively. The discontinuous vertical line indicates the base pairing between the miRNA and the wild-type target sequence, which is disrupted by the mutant nucleotide. (B) Comparison of the hsa-miR-548l target nucleotide sequence in the *FOXC1* 3'-UTR from different species. The box shows the target sequence and the underlined nucleotides correspond to the seed sequence. The alignments were carried out with ClustalW [23]. The grey background indicates the positions where the nucleotides are identical.

Fig. (3). The c.*734A>T *FOXC1* mutation specifically diminish hsa-miR-548l-dependent luciferase activity. Influence of hsa-miR-548l and hsa-miR548h on luciferase activity associated to mutations. HEK-293T cells were co-transfected with each of the different 3'-UTR *FOXC1* target constructs (wild-type, c.*734A>T or control c.*736A>T), fused to the 3' end of the firefly luciferase coding region in the pMIRTarget vector, and the pCMV-MIR vector encoding either hsa-miR-548l or hsa-miR-548h. After 48h of incubation, luciferase activity was measured as described in the Materials and Methods. RFP and LDH were detected via western blot as transfection and loading control, respectively. Error bars correspond to the SD of three independent experiments carried out in triplicate. Asterisks indicate statistical significance: p<0.001 (***). Significance was calculated by the one-way ANOVA test.

Fig. (4). The c.*734A>T *FOXC1* variants modifies both transactivation and protein levels of two coding mutations. (A) cDNA constructs encoding the indicated *FOXC1* variants were transiently co-expressed with the reporter cDNA (pGL3-basic-CXCR4 promoter-luciferase) in HEK-293T cells. The transactivation activity, expressed as a

percentage of the luciferase activity of the wild-type protein, was measured as indicated in the Materials and Methods. The protein levels of the different *FOXC1* versions present in HEK-293T cells 48 h after transfection were determined by western immunoblot using a monoclonal anti-myc antibody (Santa Cruz). Each lane contained 15 µg of total protein obtained from the cell lysates. Transfection efficiency was assessed via western immunoblot using an anti-RFP antibody (Evrogen). The sample loading control, endogenous LDH, was also detected via immunoblot using an anti-LDH antibody (Chemicon). (B) *FOXC1* levels quantitated by densitometry from bands obtained by western blot. Error bars correspond to the SD of three independent experiments carried out in triplicate. insDG: p.G447_G448insDG. NT: non-transfected cells (negative control). Asterisks indicate statistical significance: p<0.05 (*); p<0.01 (**); p<0.001 (***). Significance was calculated by the one-way ANOVA test.

Fig. (1).

Fig. (2).

Fig. (3).

Fig. (4).

Table 1. The primer sequences and PCR conditions used for *FOXC1* site-directed mutagenesis

Primer set	Sequence (5'→3')	Annealing temperature (°C)/time (s)	PCR cycles
c.*734A>T	F: GAATGAGACCTATATGTCTGGTTACTTTAATAGAGCTTTAATTAT R: ATAATTAAAGCTCTATTAAAGTAACCAGACATATAGGTCTCATT	58/15	30
c.*736A>T	F: GAATGAGACCTATATGTCTGGATTCTTTAATAGAGCTTTAATTAT R: ATAATTAAAGCTCTATTAAAGAATCCAGACATATAGGTCTCATT	58/15	30

F: forward; R: reverse. The mutant nucleotides are indicated in bold.