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In vitro and in vivo study on the secretion of the Gly367Arg mutant myocilin protein

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Purpose: Mutations in the *myocilin* gene (*MYOC*) leading to a perturbed outflow of aqueous in the trabecular meshwork (TM) has been associated with the pathophysiology of glaucoma. This study examines the expression of normal and mutant myocilin (Gly367Arg) in cultured TM cells.

Methods: Normal and mutant *MYOC* cDNA constructs were used to transfect the TM cells. In order to confirm the method of transfection, reverse trancriptase polymerase chain reaction (RT-PCR) was carried out. Further, confocal microscopic analysis was used to determine the cellular localization of myocilin protein. The extracellular nature of myocilin in the culture supernatant and cell lysates of the transfected cells was analyzed by western blot. Molecular modeling was done earlier using a knowledge based consensus method which involved threading the protein into the identified pentein fold for the COOH-terminal part. Molecular dynamics was carried out using GROMACS for the mutant model which was built using the native myocilin model.

Results: The Gly367Arg mutation causes accumulation of myocilin protein within TM cells with extensively reduced secretion contrary to wild type myocilin being characterized by intracellular localization and extracellular secretion. Further, Gly367Arg mutation occurs in a hydrophobic region which leads to burial of a charged residue. The dynamics suggests large conformational change is required to accommodate the mutation favoring aggregation of the protein.

Conclusions: Our results suggest that Gly367Arg is a potential mutation that causes malfunction of TM cells either by dominant negative effect or gain of function of mutant myocilin. The structural model suggests that the mutated myocilin could aggregate, implying the possible role of Gly367Arg in causing Primary open angle glaucoma (POAG).

Primary open angle glaucoma (POAG) is the most common form of glaucoma affecting 67 million people worldwide [1]. In India, glaucoma accounts for 1.52% of blindness [2]. A genetic approach to understanding glaucoma has been and currently is a valuable methodology and includes the useful results from the definition of the GLC1A locus [3]. However, the value of the approach is not limited to the GLC1A result or necessarily a consequence of the GLC1A result as the statement implies. The gene associated with glaucoma was identified in this locus and the gene was referred to as *TIGR* [4]. TIGR was the protein identified in TM during the induction with corticosteroids [5,6]. TIGR is now referred to as *MYOC* and the protein encoded by *MYOC* is called myocilin, which is a glycoprotein with the molecular weight of about 55-57 kDa. In the human eye, expression of myocilin is found in many ocular tissues, including TM, ciliary body, sclera, choroid, cornea, and iris [7-11]. Secreted myocilin may be required for the maintenance of normal aqueous outflow because of the presence of high levels of *MYOC* mRNA in normal and perfusion cultured TM tissues [12]. TM is the possible source for aqueous humor myocilin on the basis of myocilin secretion by the cultured TM cells when they were induced with dexamethasone [5,6]. Further, mutations in the *MYOC* gene have been associated with the dysfunction of myocilin protein in POAG [13-18]. These studies have shown that disease-causing myocilin mutants are misfolded and are highly aggregation prone. These mutants accumulate in large aggregates in the endoplasmic reticulum (ER) of TM cells where the aggregates result in the blockage of myocilin secretion. Most of the mutations associated with glaucoma are found in exon 3 of *MYOC* and to date more than seventy variations in *MYOC* have been discovered. Such mutations have been found in a subset of families with autosomal dominant juvenile onset open angle glaucoma (JOAG) and in 3-4% of adult-onset glaucoma (OAG) [19].

One of the heterozygous mutations, Gly367Arg, has been reported in various populations with severe phenotype of glaucoma. Our earlier report on Gly367Arg mutation indicates the severity of this mutation in Indian JOAG patients [20]. The present study explores the function of this mutation in cultured TM cells to demonstrate the involvement of myocilin in the pathogenesis of glaucoma.

METHODS

In vitro mutagenesis: Full-length human *MYOC* cDNA construct, subcloned in pCDNA3 vector, was kindly gifted by Dr. Darryl Nishimura, University of Iowa, Iowa City, IA. The Gly367Arg mutation (G>A) was created in *MYOC* cDNA us-

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ing inverse polymerase chain reaction (PCR) with Phusion DNA polymerase (Finnzymes, Espoo, Finland), and one of the primer pair (5'-CCG TAT TCT TGG GGT GGC TAC-3', 5'-GAA CTG TCT GTG GTA GCC AGC TCC-3') carried the mutant base A. The PCR products were digested with *Dpn*I then treated with a kinase enzyme to add the phosphate group at the 5'-end and followed by ligation. The ligated products were transformed in *E.Coli* cells and the plasmid DNA was isolated and purified using a plasmid mini prep kit (Eppendorf, Hamburg, Germany). The purified plasmid DNA was sequenced using ABI 3700 (Microsynth, Switzerland).

Transient transfection of wild type and Gly367Arg myocilin in TM1 cell line: The human trabecular meshwork cell line (TM1) was a generous gift by Dr. Mark Filla, (University of Wisconsin, Madison, WI). The cells were maintained in Dulbecco's modified Eagle's low glucose medium (Sigma, Bangalore, India) supplemented with 10% FBS, 2 mM L-glutamine, 25 µg/ml amphotericin, and gentamycin sulfate. TM cells were seeded into 35 mm dishes and cultured for 24 h. The *MYOC* plasmid DNA was mixed with the transfection reagent, Fugene6 (Roche Diagnostics, Mannheim, Germany), in the ratio of 4:2 and incubated for 15 min. The reagent complex was then added to TM cells in a serum-free medium and incubated at 37 °C at 5% CO_2. After six h the cells were replenished with serum containing the medium and incubated for 48 h, then changed to a serum-free medium 12 h prior to the collection of the culture medium.

Collection of culture medium and cell lysate: The culture medium was collected from the transfected cells and centrifuged at 10,000 rpm for five min. The medium was concentrated using the 10 kDa concentrator (Millipore, Bangalore, India) and stored at -20 °C. The cells were washed with ice cold PBS and lysed using the cell lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% TritonX-100, 0.4% SDS, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM sodium fluoride, 0.02% sodium azide, and protease inhibitor cocktail; Sigma). The cell lysates were centrifuged at 12,000 rpm for ten min. The protein concentration was estimated using the Bradford reagent (Sigma).

Reverse trancriptase polymerase chain reaction: To confirm the method of transfection RT-PCR was carried out. Total RNA was extracted from the transfected and untransfected TM cells using TRIzol and treated with DNase I. The RNA was quantified by its absorption at 260 nm and stored at -80 °C. β-Actin was used as an internal control. 0.3 µg of the total RNA was used for first strand cDNA synthesis using oligo dT primers. PCR for *MYOC* of wild type, mutant, and untransfected TM cells was carried out using exon specific

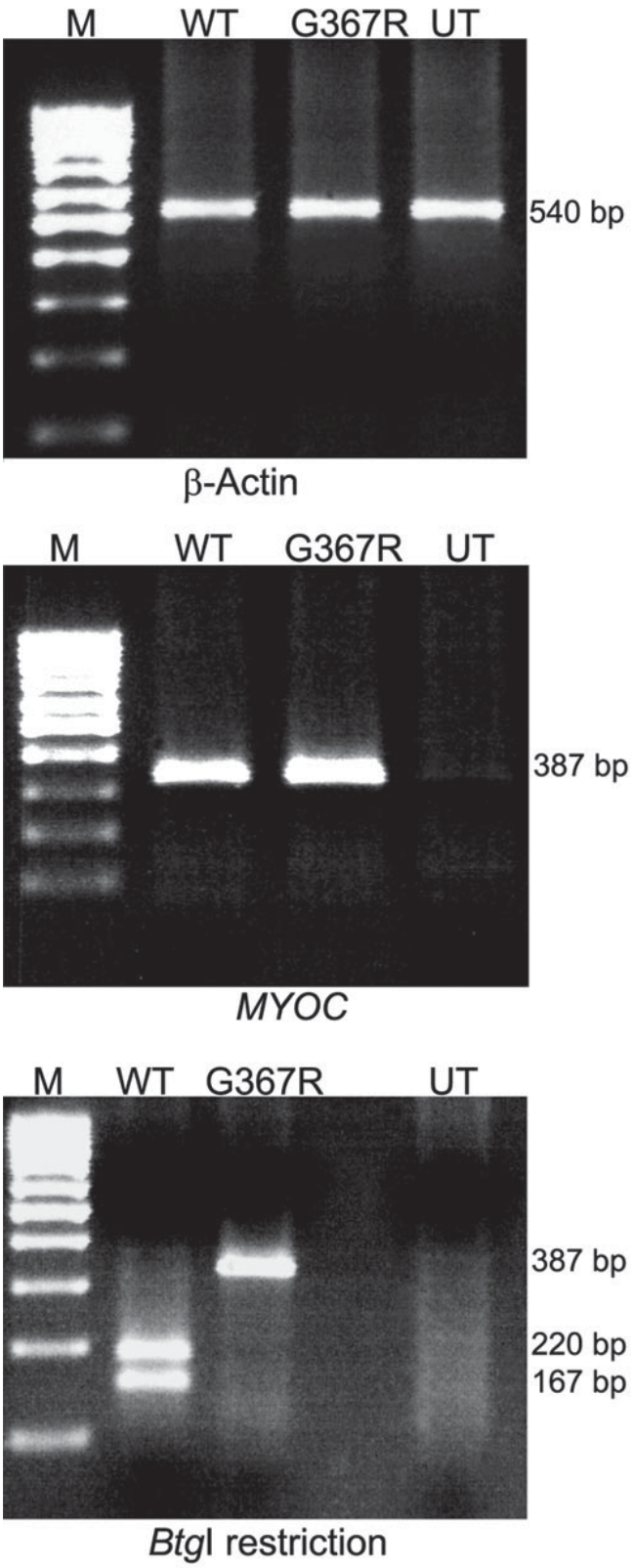

Figure 1. Reverse transcriptase-polymerase chain reaction analysis and restriction digestion. Confirmation of transfection experiment by RT-PCR and *Btg*I restriction RT-PCR was carried out using exonic primers to amplify the specific region of exon 3 of *MYOC* which resulted in 387 bp products. The left top panel showed β-actin amplification (540 bp) as an internal control in all three lanes. *MYOC* amplification was observed in the second and third lanes corresponding to TM cells transfected with wild type and Gly367Arg and was not seen in the control (untransfected). In the third lane of the bottom panel, Gly367Arg caused loss of restriction site for *Btg*I and confirmed the method of Gly367Arg *MYOC* transfection in TM cells. The wild type PCR products resulted in two bands of 220 and 167 bp as seen in the second lane. M- Marker; WT-wild type; G367R-Gly367Arg; UT-untransfected.

primers [21] to amplify the region where the mutation was created.

Restriction digestion: The presence of mutation in the PCR products was confirmed by *Btg*I restriction. The RT-PCR products of *MYOC* were restriction digested with *Btg*I enzyme to differentiate the wild type and mutant. The mutation Gly367Arg caused the loss of restriction site for *Btg*I.

Immunofluorescence staining of trabecular meshwork cells: The transfected TM cells were rinsed in PBS and fixed with 4% paraformaldehyde for 15 min. The cells were then washed three times in PBS for 10 min and incubated with rabbit anti-human myocilin antibody (1:200), a kind gift from Dr. Mike Fautsch (Mayo clinic, Rochester, NY). The primary antibody was diluted in PBS containing 0.1% TritonX-100 and the cells were incubated overnight at 25 °C. The cells were washed and incubated with biotinylated anti-rabbit IgG (1:200) for one h followed by incubation with fluorescein isothiocyanate (FITC). The cells were treated with RNase (1 µg/ml) for 20 min and counter stained with propidium iodide (PI). The dishes were mounted in mounting fluid and the immunofluorescence was analyzed using confocal microscopy.

SDS-PAGE and western blot: Culture medium and cell lysates (20 µg) of Gly367Arg as well as wild type and untransfected TM were electrophoresed in 10% polyacrylamide gel at 100 V for two h. The proteins were electrophoretically transferred to nitrocellulose membrane and blocked with 5% skimmed milk powder in PBS overnight. The membrane was washed and incubated with polyclonal rabbit anti-human myocilin antibody (1:1000 in PBS containing 1%BSA and tween-20). The membrane was washed thrice and incubated with biotinylated anti-rabbit IgG (1:1000) for one h followed by incubation with peroxidase-conjugated streptavidin (1:1000). The bands were detected using 4-Chloro-1-naphthol as the chromogen.

Molecular modeling and dynamics: The native myocilin model was built earlier [20] using a knowledge based consensus approach. In brief, it involved intermediate sequence based searches, fold recognition, and threading to identify the following structural regions from 1BOK (445-505), 1L84 (S823-S923), 1H70 (A0-A253), and 1K8Q (A92-A136) for modelling myocilin regions 1-61, xsx70-174, 180-433, and 453-504, respectively. The threading approach based on the residue based contact preference, ensured the incorporation of the known disulfide, Cys245-Cys433. The fragment joining, loop searches, and energy minimization were done using Biosym software. In order to determine the myocilin mutant model,

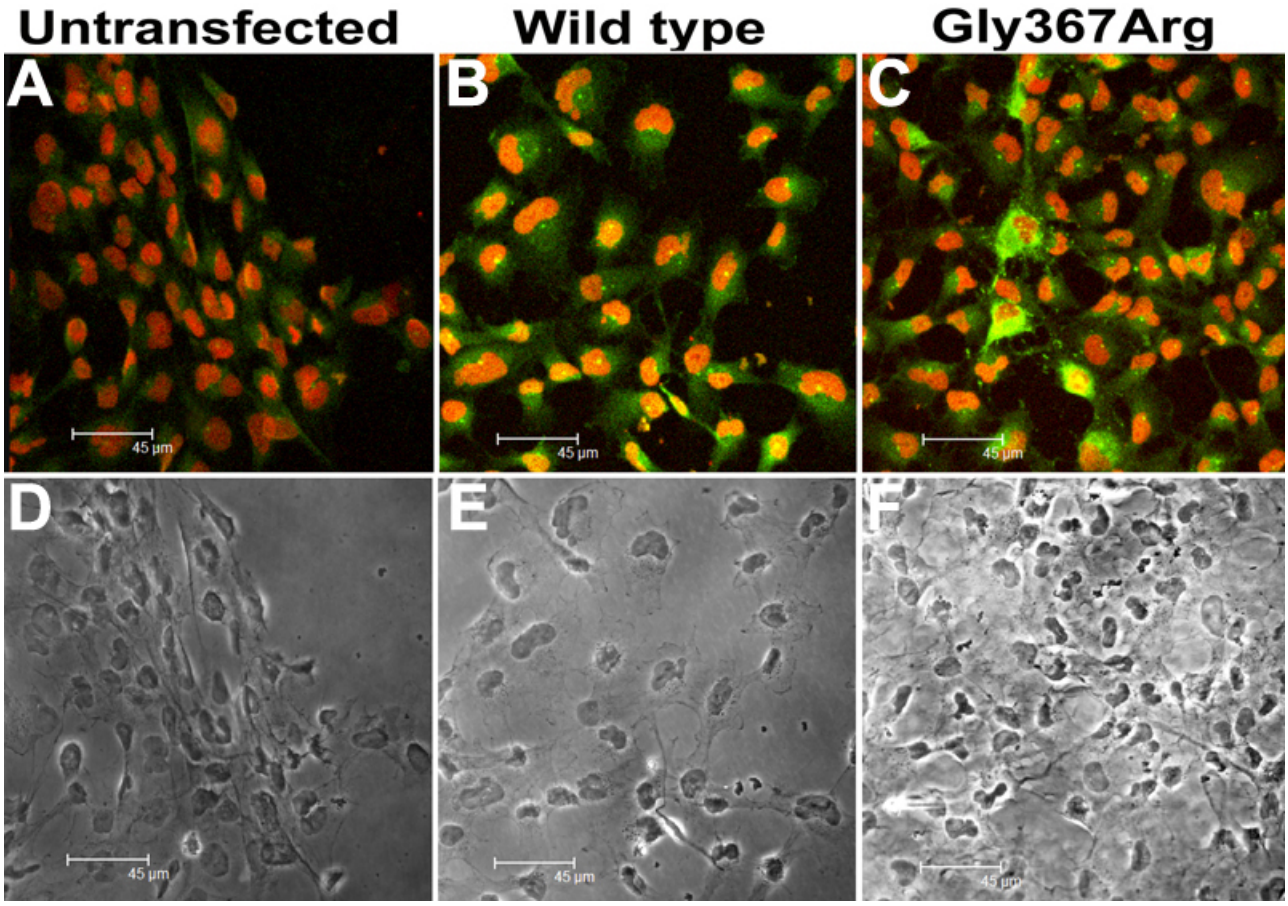

Figure 2. Confocal microscopic image of trabecular meshwork cells transfected with wild type and mutant myocilin. **A**: Untransfected TM cells showed no endogenous expression of myocilin. **B**: The TM cells showed wild type myocilin expression in the perinuclear region. **C**: Intense staining of Gly367Arg mutant myocilin in the perinuclear region of TM cells. **D**-**F**: Phase contrast images of **A**-**C**, respectively.

Glycine 367 was mutated and energy minimized. Molecular Dynamics Simulation for the native and mutant models were carried out with the native myocilin model in an eight processor parallel cluster using GROMACS [22]. The simulation was performed with the molecule in a box of water with periodic boundary condition and energy minimization with steepest descent. The dynamics trajectory was for 1 nano second (ns) with 1 femto second (fs) time step. Equilibration was achieved in 100 ps. Root Mean Square Deviation (RMSD) and Accessible Surface Area (ASA) was calculated for the trajectories with 100 ps as reference.

RESULTS

Myocilin mRNA in trabecular meshwork cells: To study the expression of a potent heterozygous mutation Gly367Arg, transfection studies were carried out to functionally determine the status of myocilin protein leading to glaucoma. The presence of wild type and mutant *MYOC* mRNA expression confirmed the technique of transient transfection before proceeding to cellular localization of myocilin (Figure 1). The *MYOC* amplification was observed in the wild type and mutant TM cells. However, there was no mRNA in the untransfected TM. In order to determine whether the amplified product was the mutant form of *MYOC*, restriction analysis was carried out. The *Btg*I restriction indicated the presence of mutant *MYOC*, which caused loss of restriction site for the enzyme (Figure 1).

Cellular distribution of wild type and Gly367Arg Myocilin in trabecular meshwork cells: Confocal microscopic analysis revealed the localization of wild type and mutant myocilin protein in the perinuclear region of TM cells (Figure 2). However, the mutant TM cells showed an intense staining pattern for myocilin. The untransfected TM cell line did not possess

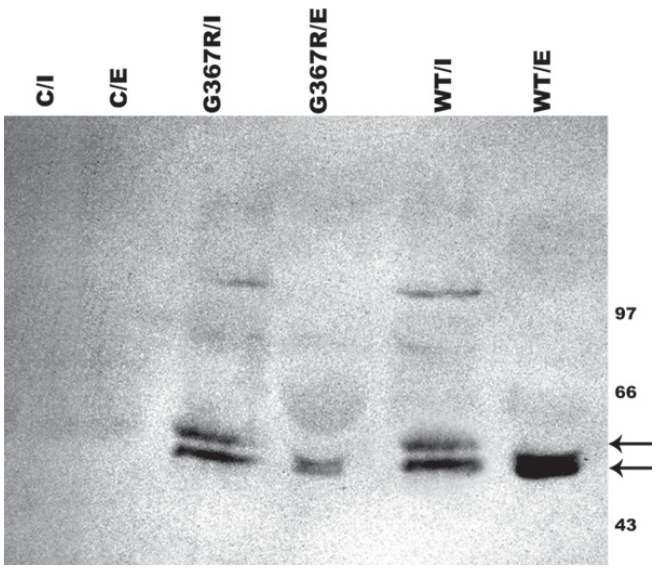

Figure 3. Western blot analysis of trabecular meshwork cells transfected with wild type and Gly367Arg *myocilin.* Myocilin was detected in the cell lysates of TM cells transfected with Gly367Arg and wild type (lanes 3 and 5, respectively), indicated by arrow. Mutant myocilin secretion was very low when compared to wild type myocilin as shown in the fourth and sixth lanes, respectively. The presence of endogenous myocilin was not observed in the untransfected cells (lanes 1, 2). I-intracellular; E-extracellular; G367R-Gly367Arg; WT-wild type; C-untransfected.

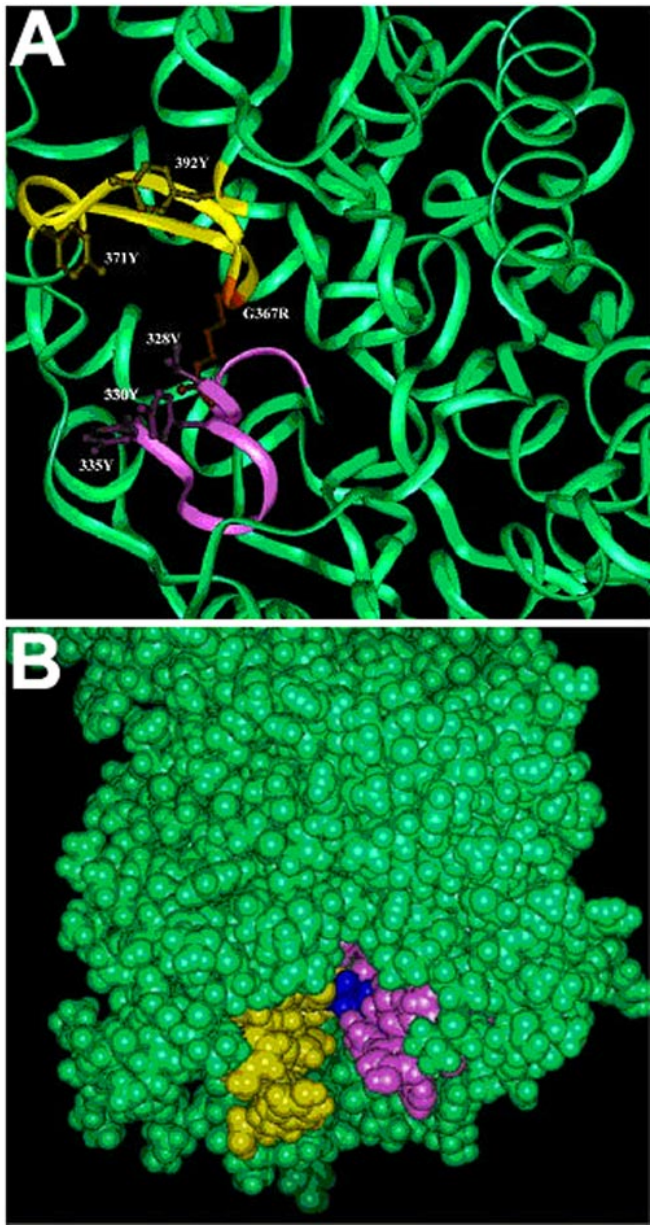

Figure 4. G367R mutation incorporated in the predicted myocilin model showing burial of the mutation in the hydrophobic region and steric hindrance which could lead to the instability and conformational change. **A**: Steric hindrance and burial of charged residue in Gly367Arg mutant. The ribbon diagram of the mutant region is shown. The mutated residue (gold color) and the neighboring hydrophobic residues (yellow and magenta colors) are shown as ball and stick model. The mutation occurs in a region where two flaps (shown in yellow and magenta) in a cavity (B). **B**: The mutated region shown in space filling model. The yellow and magenta regions correspond to the flaps shown in **A**. The side chain of arginine of Gly367Arg (blue) tends to get buried in this hydrophobic region and would therefore lead to instability and conformational change.

endogenous myocilin. The TM1 cell line has already been shown to not express the endogenous myocilin [23] and supports our data. Nonimmune staining for the transfected cells showed no staining of myocilin (data not shown). The positive TM cells expressing wild type and Gly367Arg mutant myocilin was counted using fluorescence microscopy and is presented as mean percentage of positive cells with standard deviation. The positive cells expressing wild type myocilin was 10±0.81 and mutant myocilin was 11.3±0.47.

Defective secretion of mutant myocilin in trabecular meshwork cells: Several reports demonstrate impaired secretion of myocilin protein for various *MYOC* mutations [13-18]. The present study has demonstrated the expression and secretion of Gly367Arg mutation in cultured TM cells. The western blot showed an intracellular expression of wild type myocilin with two bands of about 55 and 57 kDa. However, the secreted myocilin was observed at about 53 and 55 kDa bands. In contrast to the results with wild type *MYOC*, there was an impaired secretion of Gly367Arg mutant myocilin, which was seen as faint doublet (Figure 3). Similar results were obtained while repeating the experiment, confirming the reproducibility of the experiment.

Modeling and molecular dynamics: The mutant model shows (Figure 4) that the Arg mutation lead to the burial of the charged residue in a hydrophobic region resulting in instability and conformational change to accommodate the mutation. The molecular dynamics simulation confirmed that this region was rigid and the glycine continues to be buried as indicated by the negative accessible surface area (Figure 5). Subsequent molecular dynamics of the mutant showed that the Gly to Arg change introduces instability as seen by the RMSD analysis (Figure 6). A large conformational change is required to accommodatethe mutant so that the charged residue is not buried.

DISCUSSION

The pathogenic mechanism of glaucoma has been associated with genetic and environmental factors. However, the molecular mechanism underlying this disease has not been well understood. Myocilin is associated with the development of glaucoma and more than 70 mutations have been identified in *MYOC*. The mechanism by which myocilin mutations are involved in the pathophysiology of glaucoma has not been clearly defined. One of the risk factors for glaucoma is the elevation of interocular pressure (IOP). It has been shown that under normal circumstances, a high level of myocilin is expressed in the TM, suggesting the involvement of myocilin in the regulation of IOP. However, mutations in the *MYOC* gene may have some role to play in the development of IOP since secreted myocilin is essential for the normal outflow of the aqueous humor. It is identified that normal myocilin is secreted in the aqueous humor [24,25]. Although its secretion into the aqueous humor is unclear, many studies have shown that when the cultured TM cells are induced with corticosteroids they are able to secrete myocilin, suggesting that TM is a possible source for aqueous myocilin [5,6]. Fautsch et al. [26,27] have shown significant increase of the aqueous outflow resistance in perfused anterior segment organ cultures while perfusion of recombinant myocilin from both prokaryotic and eukaryotic expression systems.

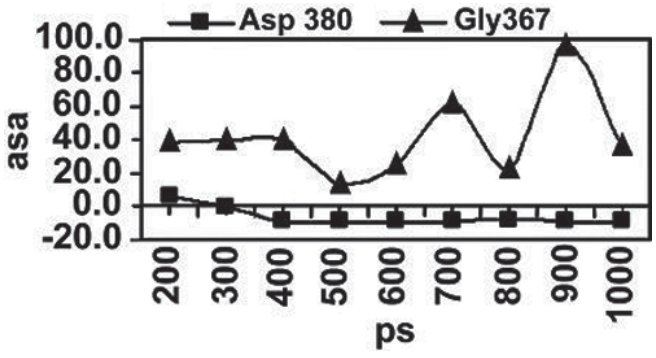

Figure 5. Burial of glycine 367. The accessible surface area (2 Å) calculated with respect to the equilibrated trajectory is shown for the molecular dynamics simulation of the native myocilin. The ASA for a non-buried residue, Asp 380, from a nearby region is shown for comparison.

The present study focus on determining the functional importance of Gly367Arg mutant protein in the pathogenesis of glaucoma. The earlier study by these authors has reported a heterozygous mutation, Gly367Arg, with severe phenotype of JOAG [20]. The patient was advised to follow up with her family members since one of her children also harbored Gly367Arg. Clinical examination of the proband's daughter revealed the development of JOAG at the age of 16. The genetic screening and counseling aided the patient in an early diagnosis led to effective treatment. The institutional review board ethics committee of the Aravind Eye Hospital, Aravind Medical Research Foundation, approved the study and tenets of the Declaration of Helsinki on human trials were strictly adhered to. Ophthalmic evaluation included best-corrected Snellen visual acuity; measurement of intraocular pressures by Goldmann applanation tonometry; anterior chamber angle evaluation by Goldmann two-mirror Gonioscope; and optic disc, retinal nerve fiber examination by 90 diopter indirect lens. The Gly367Arg mutation in *MYOC* has been reported in various populations with severe phenotype [28-30] of glaucoma. The functional importance of Gly367Arg mutation had not been studied. It is known that the secretion of myocilin is affected when there is a mutation in the *MYOC* gene associated with severe phenotype of glaucoma [13,15-17]. These studies have shown that normal myocilin was secreted from cultured cells, but very little to no myocilin was secreted from cells expressing different mutant forms of *MYOC*. It was suggested that *MYOC* glaucoma is due either to insufficient levels of secreted myocilin or to compromised TM cell function caused by congestion of the TM secretory pathway [13]. The present study demonstrates reduced secretion of Gly367Arg mutant myocilin in the cultured TM cells. A very recent report by Gobeil et al. [31] has shown non-secretion of Gly367Arg mutant myocilin in TM cells, which supports the present study's finding where partial secretion of Gly367Arg has been

observed. Therefore, it is possible that Gly367Arg mutant myocilin got aggregated in the TM cell. Furthermore, the localization pattern of the mutant protein within the TM suggests the constriction, which leads to the reduced secretion into the medium. This may be due to the aggregation of Gly367Arg mutant myocilin in the ER of the TM cells as reported for the truncated mutant myocilin, which aggregated in endoplasmic reticulum (ER) when it was co-expressed with wild type myocilin in TM cells [15]. Joe et al. [16] has reported the accumulation of mutant myocilin proteins in the ER of TM cells based on the elevated levels of protein disulfide isomerase through unfolded protein response pathway. Similarly, it was reported in another study that the mutant myocilins are misfolded in ER and bound with molecular chaperones like calnexin and calreticulin [17]. However, the present study could not predict the organelle in which the constriction of Gly367Arg occurred.

As observed in vitro, the accumulation of Gly367Arg might have blocked the flow of aqueous humor in vivo, which leads to increased IOP in the patients. Therefore, the amount of myocilin secretion was quantified in the aqueous humor of patients with Gly367Arg and of POAG patients without *MYOC* mutations (data not shown). The study observed poor secretion of myocilin in the aqueous humor of two patients who harbored Gly367Arg mutation. The low level of myocilin secretion could be due to aggregation of mutant myocilin in the TM cells as observed in vitro and the myocilin secreted in aqueous humor might be wild-type myocilin as reported by Jacobson et al for non-sense mutation Gln368Stop [13]. Furthermore, POAG patients without *MYOC* mutations showed the highest amount of myocilin secretion in the aqueous humor. However, the study was unable to determine if a difference in measurements exist between Gly367Arg mutant *MYOC* patients and POAG patients without *MYOC* mutations because normalization was not performed with an internal control and the study's sample size was small. The presence of high amount of myocilin in POAG patients without *MYOC* mutations is supported by the findings of the previous report [13] where they observed elevation of aqueous myocilin level in non-*MYOC* glaucoma patients. Further, our structural model demonstrated that Gly367Arg mutation occurs in a rigid and buried region of myocilin. Since arginine is a larger and more charged amino acid compared to glycine, this mutation would cause instability as charged residues do not tend to get buried in a hydrophobic region (Figure 4, Figure 5, and Figure 6). The longer side chain also causes steric hindrance and will

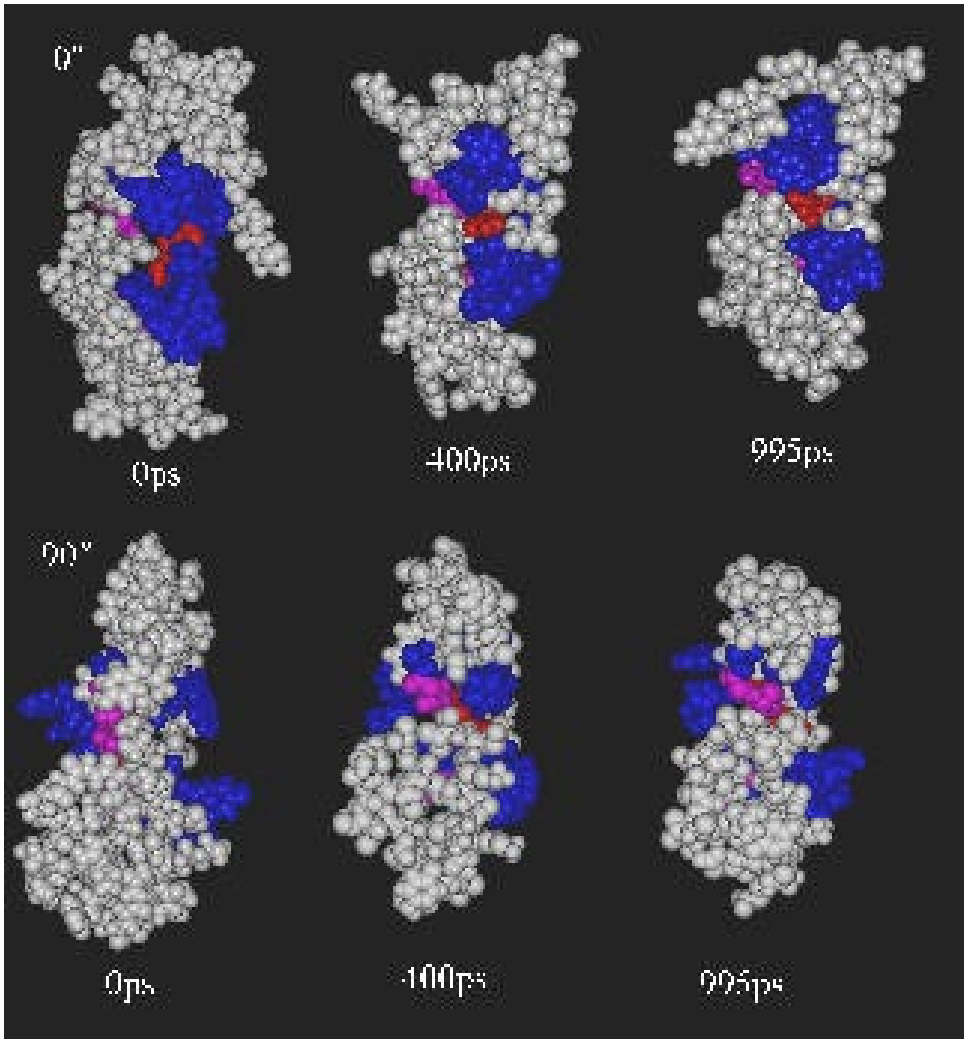

Figure 6. Instability of the arginine mutant structure. Two views of the space filling model at selected time points in the simulation are shown. The position of 367Arg is shown in red. The flanking hydrophobic regions are in blue. The 1ns simulation of the Gly367Arg mutant shows that the burial of the charged residue leads to large fluctuations throughout the model in order to try and accommodate the change. However, large conformation changes are required to prevent the burial of the charged residue.

cause a conformational change leading to exposure of the hydrophobic region and initiating aggregation of myocilin [20,32].

This study strongly suggests that Gly367Arg mutant myocilin is processed incorrectly and may aggregate in TM cells, which resultes in the poor secretion of mutant myocilin protein. This in vitro finding is correlated with the in vivo status of myocilin protein in the aqueous humor of JOAG patients with disease-causing mutation Gly367Arg.

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