AQP-9, KCNJ11 and ABCC8 Gene Variants in Open Angle Glaucoma: A Hypothesis.

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Abstract

Importance: Primary open angle glaucoma, an age-related, retinal neurodegenerative disease of unknown etiology, is treated by lowering intraocular pressure, even though elevated intraocular pressure is present in only about 60% of patients. Since we found that tolbutamide, which inhibits the opening of ATP-sensitive potassium channels, modulates aqueous dynamics with a significant increase in outflow, and since aquaporin-9 is essential for retinal ganglion cells survival, gene variants coding for the ATP-sensitive potassium channels and aquaporin-9 may participate in the development and progression of glaucoma.

Objective: To identify gene variants involved in ion and water transport in the trabecular meshwork of glaucoma donors.

Design: The study is a gene association study; since gene variants can be somatic or germline, the following genes KCNJ8, KCNJ11, ABCC8, and ABCC9, QP1, AQP4, AQP9, ATP1A1, KCNMA11,
and CLCN3 associated to ATP-sensitive potassium channels and water transport in the trabecular meshwork were sequenced from DNA isolated from trabecular meshwork of glaucoma donors.

**Setting:** The study is a gene association study carried out with samples obtained through the Cooperative Human Tissue Network of which the DNA was isolated by the technical personnel at the Lions Vision Gift eye bank and analyzed by Admera Health LLC.

**Participants:** The only criteria for the ten donors (5 male, 5 females; 70-91 years of age) in the study was a diagnosis of primary open angle glaucoma and a consent form signed at their lifetime or by responsible relatives.

**Main Outcomes and Measures:** Of several missense variants, one was found in all 10 and three were found in nine trabecular meshwork samples. All variants, whether synonymous or missense, were germline.

**Results:** The AQP9 missense variant, rs1867380 (Aca/Gca, 279T/A), was found in all 10 trabecular meshwork samples. Two common missense variants in the KCNJ11 gene, rs5215 (Gtc/Atc, 337V/I) and 5219 (Aag/Gag, 23K/E), and one missense variant in the ABCC8 gene, rs7571110 (Gcc/Tcc, 1369AS), were found in the same nine trabecular meshwork samples. Several other missense variants were found in some, but not in the majority of trabecular meshwork samples.

**Conclusions and Relevance:** Variants of the KCNJ11 and ABCC8 genes that code for subunits of ATP-sensitive potassium channels and a variant of the AQP9 gene may be implicated in the development of elevated intraocular pressure and glaucoma. Understanding how these genes impact the energetics of the neural retina may provide further insights into the pathogenic nature of these variants, as well as offer clues for developing novel therapeutic targets.

**Keywords:** Glaucoma; open-angle glaucoma; tolbutamide; ATP-sensitive potassium channels; ATP-sensitive potassium channel opener; ATP-sensitive potassium channel blocker; intraocular pressure; aqueous humor outflow.

1. **Introduction**

Glaucoma refers to a group of neurodegenerative ocular diseases that share a pathology characterized by retinal ganglion cell (RGC) degeneration [1], and progressive optic nerve atrophy that gradually lead to visual field loss and blindness. It is estimated that globally 65.5 million people are affected by glaucoma with about 5 million bilaterally blind [2]. Primary open angle glaucoma (POAG), the most common form of glaucoma, is associated with the progressive loss of RGC axons, along with supporting glia and vasculature, resulting in degeneration of the optic nerve head and loss of peripheral vision [3]. Advanced age and elevated intraocular pressure (IOP) are the main risk factors for the onset and progression of glaucoma even though lowering IOP in ocular hypertensive patients with no evidence of glaucoma reduces the development of glaucoma from 9.5% to 4.4% [4]. Nevertheless, 30-40% of patients with POAG present IOP values within the normal range [5, 6], indicating that increased IOP is not essential for neuronal degeneration. Since elevated IOP is the only modifiable risk factor, the aim of current therapeutic strategies is to lower IOP and include pharmacological treatments, surgical procedures, and laser treatment. However, in many patients RGCs’ degeneration...
continues in spite of treatments to lower IOP [7]. In fact, the risk of unilateral blindness in patients with POAG treated to lower IOP is estimated to be around 27% during a 20-year follow-up [8].

Although research is considerable, the pathological mechanisms involved in the onset and development of glaucoma are not understood. Recent research points to structural, metabolic, and functional glaucoma-driven changes in both the eye and the brain [9] and glaucoma deterioration may be already present in the eye and brain before substantial vision loss is detected clinically [10,11]. Some of the metabolic changes that are thought to underlie glaucoma pathology include calcium dysregulation [12], and alterations in glutamate and glutamine metabolism [13].

In previous work [14] we found that blockers of ATP-sensitive potassium (K\textsubscript{ATP}) channels, e.g., sulfonylureas, lower IOP whereas drugs that activate K\textsubscript{ATP} channels elevate IOP in rabbits. Using tolbutamide as a model sulfonylurea drug, we established that 0.5% tolbutamide applied topically to the eye lowered IOP and increased aqueous outflow. The finding that sulfonylurea drugs decrease IOP and modulate aqueous dynamics suggest that K\textsubscript{ATP} channels in the eye may be mutated in POAG patients.

K\textsubscript{ATP} channels are hetero-octamers consisting of four inwardly rectifying K\textsuperscript{+} channel subunits, Kir6.1 or Kir6.2, and four sulfonylurea receptors subunits, SUR1 or SUR2 or SUR2A, which belong to the family of ATP-binding cassette (ABC) transporters [15, 16]. K\textsubscript{ATP} channels, which are inhibited by intracellular ATP and activated by ADP, oscillate between open and closed states that are determined by the ratio of ATP to ADP. While open-closed state of the K\textsubscript{ATP} channels is primarily regulated by the level of ATP and ADP, several other factors appear to have modulatory effects [17,18].

A report that lactate is significantly elevated in the aqueous of glaucoma patients [19] indicates that in the eye energetics may be altered in glaucoma, especially considering that lactate is the preferred energy source for neurons [20-23]. The increased lactate in the aqueous suggests that the astrocyte-neuron lactate transfer shuttle [24, 25] is not operating effectively in the retina of glaucoma patients. In the brain and retina lactate is shuttled from astrocytes to neurons by a family of lactate/pyruvate monocarboxylate transporters (MCTs) [26, 27] with the cooperation of aquaporin-9 [27]. Even though the specific role of aquaporin-9 in the retina is not known, it is clear that aquaporin-9 is essential for the survival and function of RGCs [28-30].

Since POAG is a familial disease, genome-wide association studies have identified numerous gene variants associated with POAG, e.g., variants in the MYOC [31], the CDKN2B [32], the OPTN [33], the TBK1 [34], the ATXN233 genes, etc. However, there is no unifying hypothesis on how these gene variants may affect the metabolic neuronal ecosystem leading to neurodegeneration.

Since sulfonylureas, which are blockers of K\textsubscript{ATP} channels, lower IOP by modifying aqueous dynamics and since aquaporin-9 is essential for deliver lactate to
RGCs, we investigated whether variants of the $K_{ATP}$ channels and aquaporin-9 genes may contribute to metabolic alterations that in glaucoma may be responsible for the neurodegeneration of RGCs. Specifically, we sequenced the genes that code for the $K_{ATP}$ channels, i.e., the $KCNJ9$, $KCNJ11$, $ABCC8$ and $ABCC9$, and $AQP9$ gene that codes for aquaporin-9. Since age is a main risk factor for POAG, potential mutations could be somatic arising with age; therefore, we sequenced the exons of the $K_{ATP}$ channel genes, aquaporin-9 and several other genes from DNA isolated from the trabecular meshwork tissue (TM) obtained from donors diagnosed with POAG.

2. Material and Methods

2.1 Trabecular meshwork tissue

TM tissue was obtained through the Cooperative Human Tissue Network (CHTN) and isolated by the technical personnel at the Lions Vision Gift (Portland, OR, USA) eye bank from 10 donors (5 males, 5 females; 70-91 years of age) that had been diagnosed with POAG. The isolated TM tissue was stored for subsequent sequencing in DNA/RNA Shield buffer (Zymo Research, Irvine, CA, USA).

2.2 Sequencing

TM tissue in DNA/RNA Shield buffer was shipped to Admera Health LLC (South Plainfield, NJ, USA). DNA from TM tissue was isolated using the QIAamp DNA mini kit per the manufacturer protocol (Qiagen, Germantown, USA). Custom probes were synthesized by Integrated DNA Technologies (Skokie, IL, USA) to target the exons of the following genes: $KCNJ8$ (codes for the Kir6.1 subunit of $K_{ATP}$ channels), $KCNJ11$ (codes for the Kir6.2 subunit of $K_{ATP}$ channels), $ABCC8$ (codes for the SUR1 subunit of $K_{ATP}$ channels), $ABCC9$ (codes for the SUR2 subunit of $K_{ATP}$ channels), $AQP1$, $AQP4$, $AQP9$, $ATP1A1$ (codes for the alpha-1 subunit of Na$^+$ /K$^+$ ATPase), $KCNMA1$ (codes for the Potassium Calcium-Activated Channel Subfamily M Alpha 1), and $CLCN3$ (codes for Chloride Voltage-Gated Channel 3). Libraries were prepared with starting input of 250 ng of genomic DNA sheared using the Covaris S220 system (Covaris, Woburn, MA, USA). Libraries were combined into separate pools and targets were captured by hybridization using the Integrated DNA Technologies (Skokie, IL, USA) capture method. Quality and quantity checks were done on the Tape Station D1000 High Sensitivity and by the Qubit 2.0 dsDNA HS assay (Life Technologies, Grand Island, NY, USA). The average library size was approximately 400 bp. Sequencing was completed on the Illumina Miseq 300 Cycles to target 500x mean coverage. Data analysis, including genome alignment, was performed using the BWA software; variant discovery for the target regions was performed with the Genomic Analysis Toolkit (GATK, Broad Institute, Cambridge, MA, USA).

3. Results

In addition to several intronic and synonymous exonic variants of the gene sequenced, several germline missense variants were present (Table 1).
Table 1: Missense Variants in Trabecular Meshwork Tissue from Primary Open Angle Glaucoma Donors.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Missense Mutations</th>
<th>Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rs</td>
<td>Codon</td>
<td>Amino acid</td>
</tr>
<tr>
<td>ATP1A1</td>
<td>rs7721730</td>
<td>Atc/Gtc</td>
</tr>
<tr>
<td>AQP1</td>
<td>rs2836273</td>
<td>gGt/gAt</td>
</tr>
<tr>
<td>KCNJ11</td>
<td>rs5215</td>
<td>Gtc/Atc</td>
</tr>
<tr>
<td></td>
<td>rs1519</td>
<td>Aag/Gag</td>
</tr>
<tr>
<td></td>
<td>rs1800467</td>
<td>Ctg/Gcc</td>
</tr>
<tr>
<td>ABCC8</td>
<td>rs757110</td>
<td>Gcc/Tcc</td>
</tr>
<tr>
<td>AQP9</td>
<td>rs1867380</td>
<td>Aca/Gca</td>
</tr>
<tr>
<td></td>
<td>rs1421596</td>
<td>Gtc/Atc</td>
</tr>
</tbody>
</table>

Two missense variants, rs5215 and rs5219, of the KCNJ11 gene, which codes for the Kir6.2 subunit of the K\textsubscript{ATP} channels, and the missense variant rs757110 of the ABCC8 gene, which codes for the SUR1 subunit of the K\textsubscript{ATP} channels, were found in 9 of 10 TM samples, and the AQP9 missense variant rs1867380 was found in all 10 TM samples. No missense variants were found in ABCC9, KCNJ8, AQP4, ATP1A1, and CLCN3.

4. Discussion

In the United States with 3 million diagnosed glaucoma patients, the average cost of treating glaucoma with pharmaceuticals in 2005 was $623 per patient per year for those with suspected or early-stage glaucoma and $2511 per patient per year for patients with end-stage disease [35]. Despite these numbers, the pathological mechanism is still not understood; thus, limiting treatment options to decreasing IOP pharmacologically or surgically. Current drugs lower IOP but visual field loss continues [36], albeit at a lower rate, and many drugs are associated with significant side effects [37]. Since POAG often is not recognized by the patient until there is significant vision loss, treatment cannot be implemented too early to prevent RGC degeneration. Being able to identify patients before any RGC degeneration has occurred would allow treatment to begin before vision loss and pave the way for novel treatments.

The variants rs5215, rs5219 and rs757110 have been shown to be a risk for Type 2 diabetes (T2D) and heart disease [38-41] by increasing the hydrolysis of MgATP to MgADP [42, 43], which allows the K\textsubscript{ATP} channel to remain open.

Since the rs5215 and rs5219 KCNJ11 variants are mild gain-of-function mutations, the cellular effects have been difficult to define; for the rs5215 variant (23K/E), studies have shown a significant reduction of sensitivity to ATP (wild-type K\textsubscript{ATP} half-maximal inhibition = 71.0 ± 4.5 µmol/L ATP; 23K/E K\textsubscript{ATP} half maximal inhibition = µmol/L 120.0 ± 5.2 µmol/L ATP) as well as an increase in open probability and
reduced sulfonylurea sensitivity [44, 45]. The increase in the open probability of the channel increases the efflux of K⁺ into the extracellular space.

In considering the effect of the increased requirement of ATP to maintain the variant \(K_{ATP}\) channels in the normal open-closed oscillatory state, it is essential to consider the bioenergetics of the cell, the spatial distribution of mitochondria [46], the subcellular compartmentation of glycolytic and ATP-producing enzymes [47, 48], and the diffusion of ATP to cell-membrane compartments [49] limited by distance and impediment by the cellular cytoskeleton [45, 49]. The normal function of \(K_{ATP}\) channels is dependent on submembrane generated and not cellular bulk ATP as well as local factors such as availability of phosphocreatine [50-52]. The critical role of phosphocreatine for local modulation of \(K_{ATP}\) channels has been documented by Abrahams et al. [53] who showed that deletion of cytosolic creatine kinase, which transfers a phosphate to ADP to generate ATP, triggers channel opening in the presence of bulk ATP in cardiomyocytes.

4.1 Hypothesis. 23K/E and/or 337V/I \(K_{ATP}\) and/or 1369 A/S SUR variants; aquaporin-9 normal

In the aged TM the increased levels of ATP required to maintain the variant channel closed are not available resulting in increased efflux of K⁺, which leads to redistribution of ions, i.e. increased Na⁺, Cl⁻; and water influx into the cell and cellular swelling; the increased extracellular K⁺ is equilibrated by a decrease in water outflow to maintain extracellular osmolarity, which increases IOP without any significant harmful effects since the continuous inflow of fluid from the ciliary body and fluid outflow, even though restricted, prevents the development of a toxic milieu. In the retina, a tissue of high energy requirements, the high levels of lactate shuttled from glial cells to RGCs will generate enough ATP to normalize the open-closed state of the \(K_{ATP}\) variants while excess extracellular K⁺ is redistributed by spatial buffering [54, 55].

4.2 Kir6.2 23K/E and/or 337V/I and/or 1369 A/S SUR variants; aquaporin-9 279T/A variant

Assumption

The aquaporin variant rs1867380 does not transport lactate as efficiently as normal aquaporin-9. The lack of sufficient ATP in the aged TM results in elevated IOP and cell swelling. However, for the neural retina, which depends on lactate for its energy needs [9,21,22], there are grave consequences since aquaporin-9 is essential to transport lactate from astrocytes to neurons [27-29] in concert with monocarboxylate transporters [27], which is converted to pyruvate to produce ATP [24].

The reduced lactate in RGCs results in reduced ATP, open \(K_{ATP}\) channels, and increased efflux of K⁺. Under normal physiological conditions glutamate, which is the predominant neurotransmitter in the mammalian central nervous system, is released in the synaptic cleft and transported into astrocytes [56] where it is converted to glutamine. The transport of one glutamate is accompanied by the uptake of three Na⁺ and one H⁺ and by the release in the extracellular space of one K⁺ molecule, which is then redistributed by astrocytes [55] since sustained exposure to elevated extracellular K⁺ causes hyperexcitability and significant neuronal death [57]. In fact, even a minor
increase of extracellular potassium of 3 mEq/L decreases glutamate uptake by 40% [58].

The lack of lactate transport by aquaporin-9 limits the energy supply to RGCs, elevates extracellular K+, lactate and glutamate [59] creating a neurotoxic environment of elevated K+, glutamate [59-62], activation of the NLRP3 inflammasome cascade by the elevated K+ [63, 64], and edema [65]. In young individuals carrying the aquaporin variant 279T/A, glucose oxidation provides sufficient energy (ATP) for RGC function; however, with age the ability of neurons to utilize glucose as the energy source is lost, as it has been shown in brain [66], requiring the transport of lactose from astrocytes to meet the energetic needs of retinal neurons. The outcome of the KCNJ11, ABCC8 and AQP9 variants would be glaucoma and elevated IOP.

4.3 Normal K<sub>ATP</sub>; aquaporin-9 279T/A variant
With normal K<sub>ATP</sub> channels, the TM is not affected and the IOP will be in the normal range. However, the aquaporin-9 variant would have its effect on the retina. The decreased levels of ATP in neurons would result in open K<sub>ATP</sub> channels, K+ and glutamate accumulation in the extracellular milieu as detailed above. The outcome would be normal IOP with RGC neurodegeneration, i.e., normal tension glaucoma.

4.4 Why Sulfonylureas treatment for Glaucoma
We have shown that tolbutamide, a first-generation sulfonylurea, lowers IOP in human glaucoma subjects, and increases aqueous formation and outflow via the trabecular meshwork-Schlemm’s canal with an approximately 200% higher outflow than formation [14]. Assuming that the hypothesis that high IOP and neurodegeneration result from KCNJ11, ABCC8 and AQP9 variants, sulfonylureas would block K<sub>ATP</sub> channels, prevent K+ efflux, increase glutamate uptake, mitigate neurotoxicity [67, 68], reduce hydroxyl formation [69, 70] inhibit activation of the NLRP3 inflammasome [70], and reduce edema [71, 72].

4.5 Limitations
The authors are cognizant of the limitations of the study. The data on gene variants was obtained from TM tissue from glaucoma donors; however, we have IOP data from only one donor and we do not have a complete medical history for all donors. We have based our hypothesis on the results of the exonic sequence of several genes from 10 donors and even though 2 variants we found in 9/10 donors and one in 10/10 donors, it is possible that sequence analysis of a large population may show different results. It is also important to note that aquaporin-9 is not well studied; the AQP9 rs1867380 variant has been reported only in two publications that implicate it in the level of fetal hemoglobin in sickle cell disease [73,74]. For the hypothesis presented here, we have assumed that the AQP9 variant is not as effective as shuttling lactose to RGCs, which remains to be proven.

5. Conclusions
We have presented a hypothesis for the development of glaucoma based on the effect of sulfonylureas on the IOP of rabbits, on the effect of tolbutamide on human glaucoma patients [14], and on the presence of KCNJ11, ABCC8 and AQP9 gene variants in the TM of donors diagnosed with glaucoma. Limitations aside,
the hypothesis can be platform for studies to prove, disprove or modify the hypothesis. If the AQP9 variant is responsible for the development of glaucoma, it can be used to design a genetic test to identify patient at-risk of glaucoma, institute treatment early while forgoing treatment of ocular hypertensive patients that do not have the AQP9 variant, and design gene therapeutic strategies.

Author Contributions
Both authors contributed equally to the study reported here.

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Conflicts of interest
The authors are co-inventors of patent No: 10,780,068 “Methods and compositions for improving eye health”, and co-inventors of a Continuation-in-part application by the same title, application No.:16/938,628.
Nino Sorgente is the co-inventor on Patent No: 5,965,620 and 5,629,345 “Methods and compositions for ATP-sensitive K+ channel inhibition for lowering intraocular pressure”.
Nino Sorgente is President of the newly incorporated Elements Pharmaceuticals.

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