Book Chapter

CYP1B1 Gene Alteration Associated with Cancer While Mutation (p.Gly61Glu) Alters Microglia Activities and Promote Glaucoma Progression

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List of Abbreviations

AOAG-Adult Open Angle Glaucoma; CSF-Cerebrospinal Fluid; CDC-National Centres for Disease Control and Prevention; CGRN-Childhood Glaucoma Research Network; CRISPR Cas-Clustered Regulatory Interspaced Short Palindromic Repeats (CRISPR) and (Cas) Complex Sensing Systems referred as (CRISPR-Cas); CYP1B1-Cytochrome P450 Family I Subfamily B Polysaccharide Member I; GWAs-Genome-Wide Association Study; IOP-Intraocular Pressure; JOAG-Juvenile Open Angle Glaucoma; LC-Lamina Cribrosa; PCG-Primary Childhood Glaucoma is further classified into Primary Congenital Glaucoma; POAG-Primary Open Angle Glaucoma; TM-Trabecular Meshwork; WGA-World Glaucoma Association; WHO-World Health Organization; ONH-Optic Nerve Head

Introduction of Visual Systems

The organization of this chapter follows the order of neural events as they occur in the visual systems, beginning with the anatomy and physiology of the human eye and ending with the primary visual cortex, which projects to the visual association region where visual information is elaborated and synthesized with experiences and memory. Vision and nature of visual perception is the most highly developed and versatile of all sensory modalities in human beings. Vision is an active process and tissues of the human eye directly impact vision are cornea, lens, retina, and optic nerve. Vision commences with the formation of an image of the external world on the photoreceptive retina. The retina encodes visual information in the discharge of neurons which project to the brain in the optic nerve. It is the fibers of the optic nerve undergo hemi-decussation as two optic nerves converge to form the optic chiasma and project lateral geniculate nucleus of the thalamus. Humans that are blessed with normal vision largely react to the world as they see it, rather than to feel or its sound or smells. We learn to interpret the images we see. We associate particular properties with specific visual objects and have remarkable capacity to identify objects, including the perception of form, space and motion, particularly other people, from quite subtle cues. The importance of the way the brain uses visual cues to form its internal representation of an object is illustrated by many visual illusions as best known is the Muller-Leyer illusion. Vision in humans involves the detection of a very narrow band of light ranging from 400 to 750nm in wave length. The shortest wave-length are perceived as blue and the longest as red. The eye contains photoreceptors that detect light but, before the light reaches the receptors responsible for this detection, it has to be focused onto retina (200μ thick) by the cornea and the lens as shown in [1].

Organization and Functions of the Visual Cortex

Thalamo-cortical neurones in turn project to the primary visual cortex of the occipital lobe where visual perception occurs [1].The visual system is concerned with image perception, which involves a combination of four events in summary: (a) refraction of light rays by the lens and cornea; (b) conversion of electromagnetic energy of light rays by retinal photoreceptor cells into the nerve impulses; (c) transmission of generated action potentials from the receptor cells of the retina to the central nervous system and to the visual cortex in the occipital lobe; and (d) perception of visual images in the primary and interpreted in the associational secondary visual cortices [2]. The optic nerve includes the visual fibers from the retina to the optic chiasm in the central visual pathway. The optic tract contains axons which carry information relating to the contralateral half of the field of vision. Optic tract fibers travelling between the chiasm end in the lateral geniculate nucleus of the thalamus. Where some fibers are known to reach the superior colliculus. It is the thalamic cells which project axons to the visual cortex via geniculocalcarine (optic radiation) fibers. The visual field is the area in the outside world that can be seen. Thus, the retinal field conversely, is focused representation of the visual field. The monocular visual field is the lateral portion of the visual field that is perceived in only one eye. Whereas the binocular visual field is seen in both eyes as they simultaneously focus on a single object. It is the light rays from an object that strike the identical retinal points in both eyes. This results in the images from both eyes that are cortically merged into one image. Binocular vision is involved in depth perception. The optical disturbances affect image formation, whereas lesions interrupting visual fibers results in different visual field losses [1].

The Eye Receptor and Neuronal Functions, Organization of the Retina

The cellular layer of the retina is in the posterior portion of the eyeball. The retina is the sensory region of the eye, a specialized growth of the brain which represents a complex arrangement of layers of cells such as photoreceptors (rods and cones), the first-order neurone, or bipolar cells within the retina, and second-order neurone, or ganglion cells constitutes the optic nerve (the papilla or optic disc). The layers between the retina surface and the receptor cells contain a number of excitable cells, called bipolar, horizontal, amacrine and ganglion cells. Information is transferred from the photoreceptors to bipolar cells and, to ganglion cells with greater convergence for rods than for cones. Each retina contains about 100 million rods and 3 million cones and ganglion cells are only 1.6 million. Therefore, an average of 60rods and 2 cones converge on each ganglion cell to the brain. The retina contains interneurons that are known as horizontal cells and amacrine cells. These modulates transmission between bipolar cells and ganglion cells, respectively. The ganglion cells are the neurones that transmit impulses to the rest of the central nervous system (CNS) via axons in the optic nerve. The ganglion cells are further divided into two main groups: P cells and M cells. It is the P cells that receive central parts of their receptive fields from one or two types of colour specific-cone. Whereas M cells receive inputs from all types of cone, and are not colour selective but, yare sensitive to contrast, and movement of images in the retina. This division of P and M cells appear to be maintained throughout the visual pathways and are involved in visual perception. These cells are excited by the vertical bipolar interneurons which are in between the receptor cells and the ganglion cells. In addition, this complex structure comprises of two groups of interneurons (horizontal and amacrine cells) that function by exerting their influence in a horizontal manner, by causing lateral inhibition on surrounding synaptic connections between receptor cells and bipolar cells, and bipolar cells and ganglion cells, respectively. There are three distinct types of retinal ganglion cells, designated W, X, and Y cells and each of these serves a different function in their respective fields. The transmission of rod vision is by the W

cells; visual image and colour by the X cells and Y cells are functionally transmit instantaneously changes in the visual image [1].

A series of photochemical reactions and physicochemical changes, retinal photoreceptors transduce light energy into electrical energy (changes in membrane potential). The ganglion cells are the first cells to generate action potentials in axons and are usually spontaneously active. Ganglion cell axons coverage at the optic disk to form the optic nerve. The human retina consists of two distinct types of photoreceptors, called rods, and cones, that are functionally and structurally different. Rods respond to dim light and cones respond in brighter conditions and can distinguish red, green or blue light. The rods and cones are found in the deepest part of the retina, and light travels through a number of cellular layers to reach these photoreceptors. The light - sensitive-chemical is called rhodopsin found in the rods; whereas the light-sensitive-chemical is called cone pigments or colour pigments found in the cones. Each of the photoreceptors contain molecules of the visual pigments (rods; rhodopsin; cones; erythrolabe (red), chorolabe (green) and cyanolabe (blue): these absorb light and trigger receptor potentials which, lead to hyperpolarization of the cells. Each eye contains approximately 126 million photoreceptors (120 million rods and 6 million cones) but only 1.5 million ganglion cells. Therefore, there is a substantial amount of convergence of receptor and bipolar cells onto ganglion cells, but this is not uniform across the retina. At the periphery, there is a large amount of convergence, but, in the region of greatest visual clarity known as the forvea centralis with very high density of cones about 35,000 of them and no rods. Both rods and cones are sensitive to light energy in light rays of different wavelengths, mediate vision under different wavelengths, mediate vision under different light conditions, and contain different visual pigments [1].

The Organization of the Central Visual Pathways

The axons of the retinal ganglion cells assemble at the optic disc (blind spot) and pass into the optic nerve which enters the cranial cavity through the optic canal. The optic nerves from the two eyes join at the base of a skull at a structure called the optic chiasma. It is from the lateral geniculate nucleus, third-order thalamocortical neurons project through the retro-lenticular part of the internal capsule forming the optic radiation, which terminates in the primary visual cortex of the occipital lobe. The rest of the occipital lobe constitutes the visual association cortex. It is concerned with interpretation of visual images, recognition, depth perception and colour vision. There is precise communication between the retina and visual cortex because of the importance of the macula lutea in vision. Which is represented by disproportionately large volumes (relative to its size) of the lateral geniculate nucleus and visual cortex. It is within the visual cortex that the macula is represented most posteriorly, in the region of the occipital lobe. The visual images are focused in the macula lutea for clarity and colour analysis. The fovea centralis is responsible for the visual acuity, and sharp visual colour perception [1].

Glaucoma, is a heterogeneous disease and leading cause of irreversible blindness worldwide. It is a group of chronically neurogenerative ocular disorders of the optic nerve and different types of glaucoma been classified traditionally into four categories based on specific criteria for example primary open-angle glaucoma (POAG), primary congenital glaucoma (PCG), primary normal-tension glaucoma, and primary angle-closure glaucoma (PACG). Evidence from first published article on the effects of CYP1B1 mutations on primary astrocytes and microglia of rats, with human skin mesenchymal stem cells were investigated by study of [3].

Microglia's Loss of Activity is Associated with CYP1B1 Mutation

The primary congenital glaucoma (PCG) in association with variant (p. Gly61Glu) in CYP1B1 gene has been investigated. The direct impact of the mutation enhanced microglia's loss of activity and facilitated glaucoma progression in Saudi Arabia [3]. The following parts will explain experimental parts and discussion for this conclusion.

Background

Defects in *CYP1B1* have been associated with immature eye development causing developmental anomalies, such as trabecular meshwork dysgenesis [4]. Although no data is available for other parts of Saudi Arabia, we suspect that *CYP1B1* mutation is still the most common mutation linked to PCG in Saudi Arabia based on the available studies [4,5]. The PCG variant p.Gly61Glu in *CYP1B1* has been identified as the major disease-associated mutation in Saudi Arabia, representing 63% of all cases [4,6]. It is not well understood how the *CYP1B1* mutation p.Gly61Glu damages the optic nerve or leads to blindness during disease progression. The optic nerve is supported by glial cells, or the neuroglia, which play a vital role in its maintenance. These cells occupy half of the brain space and are considered non-neuronal cells in the CNS. They are classified into three major cell types: astrocytes, oligodendrocytes and microglia [7]. Astrocytes are known to promote neuronal survival, myelination and synaptogenesis. In addition, they regulate neurotransmitters, ion exchange and the blood-brain barrier [8,9]. Microglia are known to eliminate microbes, dead cells, cellular debris, excess synapses, protein aggregates and other particulates [10]. The molecular aspects and consequences of having the *CYP1B1* p.Gly61Glu variant in optic-nerve supporting cells are not understood. This study shows the effects of the *CYP1B1* mutation p.Gly61Glu (G61E) on the supporting cellular components of the optic nerve, such as glial cells. We focus on microglia and astrocytes (which are parts of the glial cell population) and their responses to *CYP1B1* mutation. Metabolism of endogenous and exogenous compounds

depends on cytochrome P450 activity [11,12]. The CYP protein was previously called 'cytochrome P450' following the discovery of a coloured pigment in the cell that had a 450 nm wavelength spectrum when reduced and bound with carbon monoxide. The name continues to be misused as these proteins are not true cytochromes. They are more appropriately called 'heme-thiolate monooxygenases' [13]. CYP enzymes are stimulated by aromatic hydrocarbon receptors (AhRs), and they consist of a large number of proteins. They are involved in the metabolism of xenobiotics and endogenous compounds such as oestradiol [14]. They are also responsible for the biotransformation of most foreign substances and drug detoxification [15]. CYP expression is affected by many factors, such as sex, age, and genetic polymorphisms. The expression can also be influenced by stimulation from xenobiotics, through modulation by cytokines and hormones, and during disease states [16].

Materials and Methods

Primary Cell Extraction from Animals

Healthy two-day-old male Sprague-Dawley rats weighing 5–6g were obtained from the King Saud University (KSU) animal facility in the College of Pharmacy. All animal involvements were ethically approved by King Abdullah International Medical Research Center Review Board, RC17/038/R. The isolation of astrocytes and microglia from the brains of these animals was performed using Hong Lian's protocol [17]. The rats were decapitated, and their heads were placed in a 5% foetal bovine serum (FBS) (Catalogue #10099141, Gibco, United States of America [USA]), 1% Pen-Strep antibiotic (Catalogue #15140–122, Gibco, USA) and DMEM (Catalogue #11885–084, Gibco, USA) medium. The protocol was carried out in a sterile class II biosafety cabinet. The meninges were removed using forceps, and the cortices and hippocampi were collected. The tissues were homogenised and divided into two prepared tubes that contained trypsin, DNase, phosphate buffer saline (PBS), 10% FBS, 1% GlutaMAX (Catalogue #35050–061, Gibco, USA), 1% Pen-Strep antibiotic, DMEM, trypsin (Catalogue #12604–013, Gibco, USA), DNase (Catalogue #LS002007, Worthington Bio-

chemical Corporation, USA) and PBS (Catalogue #10010–031, Gibco, USA). These tubes were vortexed and then incubated with 5% CO_2 at 37℃ for 20 mins (mixed carefully every 5 mins). 100µm and 40µm cell strainers were used, and centrifugation was performed at 400x g for 5 mins. Each tube was washed twice with PBS and RBC lysis buffer. Pellets were taken and washed with 0.9M of sucrose. Again, centrifugation was performed at 600x g for 5 mins to discard the supernatant. The pellets were pelleted on culture flasks containing 10% FBS, 1% GlutaMAX, 1% Pen-Strep and advanced DMEM. The flasks were vigorously tapped for a few days into the culture, which typically releases microglia from the culture flask while leaving astrocytes attached. Finally, the medium containing the microglia was placed into new flasks along with GM-CSF (Catalogue #PHC2013, Thermo Fisher Scientific, USA) growth factors. Additionally, new media with 10% FBS were added to the old flasks containing astrocytes. Proper ethical approvals were obtained for this project from KSU and the King Abdullah International Medical Research Center (KAIMRC) in Saudi Arabia. The purity of the microglia was 91%, whereas the purity of the astrocytes was 93.9%.

CRISPR Editing of Target Cells

A custom-designed CRISPR kit (Origene, Rockville, MD, USA) for *CYP1B1* (c.182G>A, p. Gly61Glu, a CRISPR pCas-Guide) was used, as previously described in other studies [18,19]. In the pCas-Guide, the following modified sequence was used: For transfection, the GenMute Reagent (Catalogue #SL100568, SignaGen, USA) was used. Normal microglia, astrocytes and stem cells (Hs27) were inoculated in groups of 300,000 cells in T25-flasks in 10% FBS at 37℃ overnight. The next day, cells were starved in 1% FBS for 24 hrs. On the third day, the *CYP1B1* mutation was introduced into the target cells—that is the normal microglia, astrocytes and Hs27—separately via GenMute reagent using the CRISPR kit. The working solution of GenMute transfection was prepared by adding the CRISPR construct of the mutation. A second working solution was also prepared separately for the control, mock mutation (normal DNA sequence), which did not result in any amino acid changes in the target gene. All flasks were incubated for 4 hrs at 37℃ after

transfection. The media were then replaced with a fresh advanced DMEM-conditioned medium, which was the same medium used in the isolation step. The transfection efficiency of the reagent is around 99%, which was validated in our previous study [20]. However, damage to the DNA after transfection was not evaluated. Therefore, potential false positive results due to CRISPR activity should be considered when interpreting the results.

Proliferation Assay

Primary microglia and astrocytes obtained from rats were inoculated in groups of 5,000 cells per well in 96-well plates in 10% FBS at 37℃ overnight. The next day, all cells were starved with DMEM (Catalogue #21885025, Thermo Fisher Scientific, USA) containing 1% FBS and were incubated overnight at 37℃. All cells were nourished with complete growth-specific media on the assay day. The *CYP1B1* gene was manipulated in both the microglia and the astrocytes. The control cells received mock manipulations. After 24 and 72 hrs, both the *CYP1B1*-edited microglia and astrocytes were compared with the control, mock-edited cells by measuring their metabolic activity using the MTT Cell Proliferation Assay (Catalogue #V13154, Thermo Fisher Scientific, USA). The company protocol was followed for assay performance. The signal was read via SpectraMax reader with SoftMax Pro v.6.2.1 software. Furthermore, similar conditions were applied to a commercial Hs27, human mesenchymal stem cell (fibroblast) line (Catalogue #CRL-1634, ATCC, USA), and then the MTT Assay was performed.

Nicotinamide Adenine Dinucleotides Phosphates (NADP) Reduction Assay

For the assay, the amount of NADP and NADPH was determined in the biological samples. In the presence of NADP+ and NADPH, the enzyme reductase reduces a proluciferin reductase substrate to form luciferin (#G9081, Promega, USA). The total estimation of NADP/NADPH was reported. The measurement took place after 24 and 72 hrs of *CYP1B1* gene manipulation on rat microglia, astrocytes and Hs27 using the SpectraMax M5 reader and software (SoftMax Pro v.7.0.2).

Phospho-kinase Array

After 24 hrs of *CYP1B1* gene manipulation or mock manipulation in both microglia and astrocytes, cell lysates were collected from *CYP1B1*-edited microglia and astrocytes in addition to the collection of normal control lysate. All the lysates were prepared using RIPA solution (Catalogue #89901, Thermo Fisher Scientific, USA), which contains protease and phosphatase- inhibiting mini tablets (Product #88669, Thermo Fisher Scientific, USA). Protein quantitation was measured using a Microplate BCA Protein Assay Kit (Product #23252, Thermo Fisher Scientific, USA). A total of 300x g of protein was used for each array membrane. The phospho- kinase array buffers were prepared and mixed with samples according to the manufacturer instructions (Catalogue #ARY003B, R&D system, USA). Signals were detected with chemiluminescent substrate (Product #34077, Thermo Fisher Scientific, USA) and were read using a ChemiDoc MP Imaging System and associated software (Image Lab v.5.1). The analysis and estimation of proteins were both conducted using the Image J Program (v.1.5.2a). The graph bars were made using GraphPad PRISM v8.

Bio-plex Panel for Inflammatory Cytokines Assay

The culture media of the *CYP1B1*-edited and control (mock) cells were collected for both astrocytes and microglia between the fifth and eighth days after manipulation, when the cells were confluent. The collected fractions of media were frozen at -80℃ until use. The protocol of the Bio-Plex Pro Inflammation Panel 1 Assay was followed according to the manufacturer instructions (Catalogue #171-AL001M, Bio-Rad, USA). The samples were mixed separately with the kit's beads in 96-well/ELISA plates. Each sample was inoculated in duplicates. Detection antibodies and streptavidin-PE were added as instructed with proper washing. The lower and upper limits of quantitation (LLOQ and ULOQ) were imputed from the standard curves included in the kit. The plate was covered with aluminium foil until it was installed in the BioPlex three-dimensional (3D) Suspension Array System (xPONENT software v.4.2.1441.0). The samples tested were conditioned with media collected from mutated microglia, control micro- glia, mutated astrocytes and control

astrocytes. Conditioned media were collected from two independent experiments, and each sample was plotted in two different wells.

Protein Extraction and Immunoblotting (Western Blot)

A minimum of 500,000 cells were used per assay. Similar to previously published protocols [20], the cells from *CYP1B1*-edited microglia and astrocytes were lysed, and the proteins were quantified. Likewise, the mock-edited microglia and astrocytes were lysed, and the proteins were quantified using a Microplate BCA Protein Assay Kit. Immunoblot analysis was performed by loading 20µg of protein samples on an SDS page of 10–20% gels (Life Technologies). Transfers were performed on wet transfer cells (Bio-Rad) with PVDF membranes (EMD Millipore). The antibodies used were anti-β-actin, internal control (Catalogue #3700, Cell Signaling Technology, USA), RXRα (Catalogue #3085, Cell Signaling Technology, USA), RXRβ (Catalogue #8715, Cell Signaling Technology, USA), RXRγ (Catalogue #5629, Cell Signaling Technology, USA) and melatonin (*MTNR1B*) (Catalogue #ab203346, Abcam, USA). The secondary antibodies were goat anti-mouse (Catalogue #926–32210, Li-Cor, USA) and goat anti- rabbit (Catalogue #926–68021, Li-Cor, USA).

Acquisition of Florescence Activated Cell Sorting (FACS) Techniques

As previously described [21], samples containing live dissociated cells were stained for dead cell apoptosis using the annexin V Alexa Fluor antibody and propidium iodide kit (Thermo- Fisher #V13241) and were then analysed with the FACS CANTO II (BD Biosciences) Fluorescence-Activated Cell-Sorting Machine. Twenty-four hours before staining, the cells were manipulated with a mock mutation or the *CYP1B1* mutation (p.G61E). The background was deducted and defined with unstained cells.

Statistical Analysis

All experiments have been repeated three independent times, except for the phospho-kinase assay, which was performed

twice. An unpaired t-test was used as the default for all comparisons. Each assay was normalised, and a simple comparison was made between the control and the test for each reading. An asterisk (*) was given when the p-value was less than or equal to 0.05.

Results and Figures

Decreased Proliferation Detection on CYP1B1 Mutated Cells

The mutated astrocytes and microglia, showed hyperactivity and an increase in proliferation of more than 10% within 24 hrs after introducing the CRISPR-edited *CYP1B1* (c.182G>A, p. Gly61Glu), compared to the control (Figure 1A; $p < 0.05$). Two days later, the microglia proliferation activity reduced by 60% in the CRISPR-mutated cells, compared to the control (Figure 1B; $p < 0.05$). The differences between the astrocytes and the control were not statistically significant ($p = 0.214$). In addition, the assessment of the human fibroblast (mesenchymal stem cells, Hs27) metabolic activities revealed no significant changes in the activity of the *CYP1B1*- mutated cells, in contrast to the control, within 24 hrs (Figure 1C; $p = 0.482$). After 72 hrs, the reduction in proliferation (metabolic) activities reached 40%, compared to the control, as shown in Figure 1C ($p < 0.05$).

Death of Mutated Microglia over Time

We observed a tendency in the mutated microglia to die after three or more days in culture. Changing the media every day, prevented premature death due to the toxicity of too much debris. The flow cytometry analysis with annexin V (pre-apoptosis detection), which was conducted 24 hrs after *CYP1B1* or mock manipulation, showed 71% pre-apoptosis, compared to 13% in the control cells (Figure 1D).

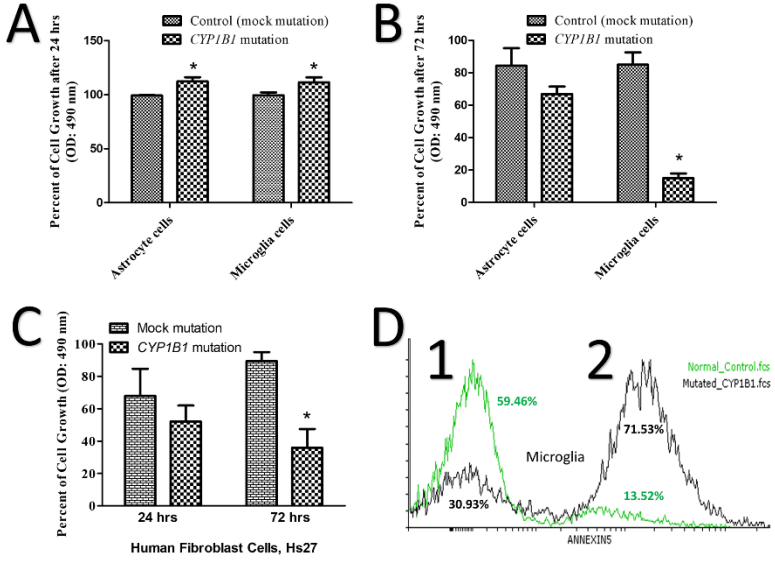

Figure 1: Mutant microglia show less cell growth and more apoptosis than the control. (A) A 10% increase in the growth of *CYP1B1* mutated astrocytes and microglia after 24 hrs. (B) After 72 hrs, growth was decreased in *CYP1B1* mutated microglia by 60%, but not in astrocytes. (C) The percentage of metabolic activities of the *CYP1B1* mutated mesenchymal stem cells line (Hs27) after 24 hrs and 72 hrs. (D) The assessment of microglia pre-apoptosis using an annexin V assay shows 71.53% pre-apoptotic cells in the mutated cells compared to 13.52% in the healthy control. An asterisk (*) is displayed when the p-value was less than or equal to 0.05; the number of experimental repeats (N) was three.

Changes in NADP/NADPH Expression Detected in CYP1B1 Mutated Cells

The mutated microglia exhibited a significant 30% decrease ($p < 0.05$) in NADP/NADPH activity compared to the control (Figure 2A). In addition, the mutated *CYP1B1* genes in both the rat astrocytes and the human mesenchymal stem cells (Hs27), revealed no statistically significant changes in NADP/NADPH activity within 24 hrs, compared to the control (Figure 2B and 2C; $p = 0.310$). Similarly, the NADP/NADPH activity did not change after 72 hrs for either the mutated astrocytes or the Hs27, compared to the control (Figure 2B, $p = 0.6761$ and 2C, $p = 0.392$).

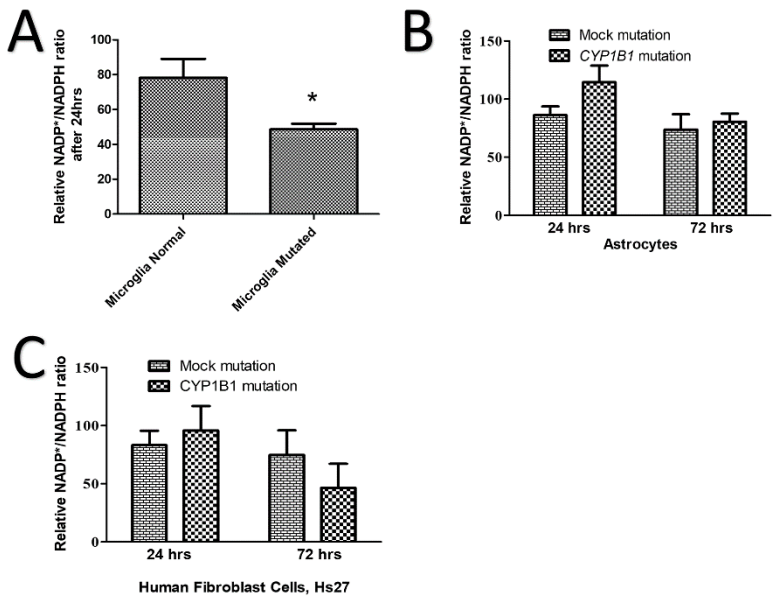

Figure 2: Mutated microglia show less reducing equivalent ratios than the control. (A) The normalised percentage of reducing equivalent ratio $NADP^*/NADPH$ decreased by 30% in mutant microglia compared to the normal control after 24 hrs. (B) The normalised percentage of the reducing equivalent ratio of $NADP^*/NADPH$ comparing the control (mock) astrocytes to mutated ones after 24 and 72 hrs, showing no significant difference. (C) The normalised percentage of the reducing equivalent ratio of $NADP^*/NADPH$ comparing the normal Hs27 to the mutated ones after 24 and 72 hrs, showing no significant difference. An asterisk (*) is displayed when the p-value was less than or equal to 0.05; N = 3.

Deferential Expression of Phosphorylated Proteins

The mutated microglia and astrocytes, showed differentially expressed phosphokinases, such as EGFR, ERK 1/2, GSK3 a/b, STAT3 and STAT5, compared to the control (See Figure 3 and 4). All of the above proteins are involved in proliferation-stimulating pathways [22]. The mutated microglia significantly ($p < 0.05$) expressed 11 of the 29 proteins tested, in comparison to the control cells (Figure 3). In addition, the mutated astrocytes significantly ($p < 0.05$) expressed 10 of the 29 proteins tested, in comparison to the control (Figure 4).

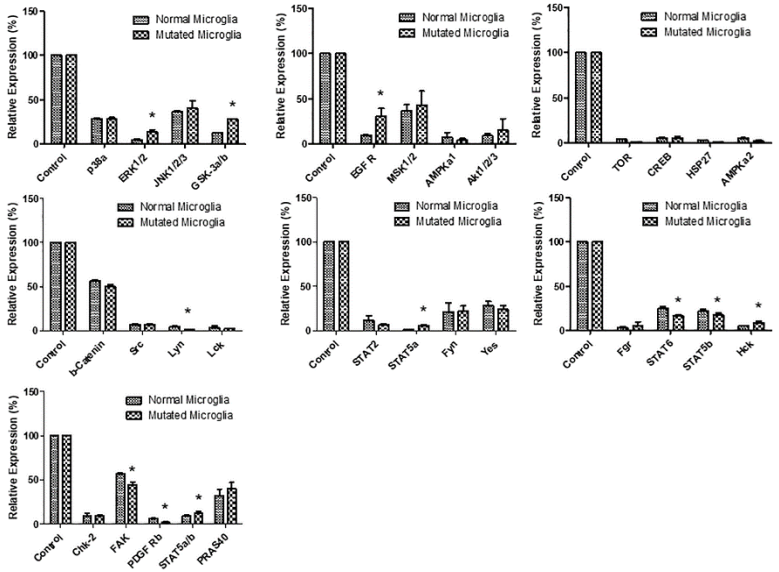

Figure 3: Normalised phospho-kinase expression of mutant microglia compared to a normal control. An asterisk (*) is displayed when the p- value was less than or equal to 0.05; N = 2.

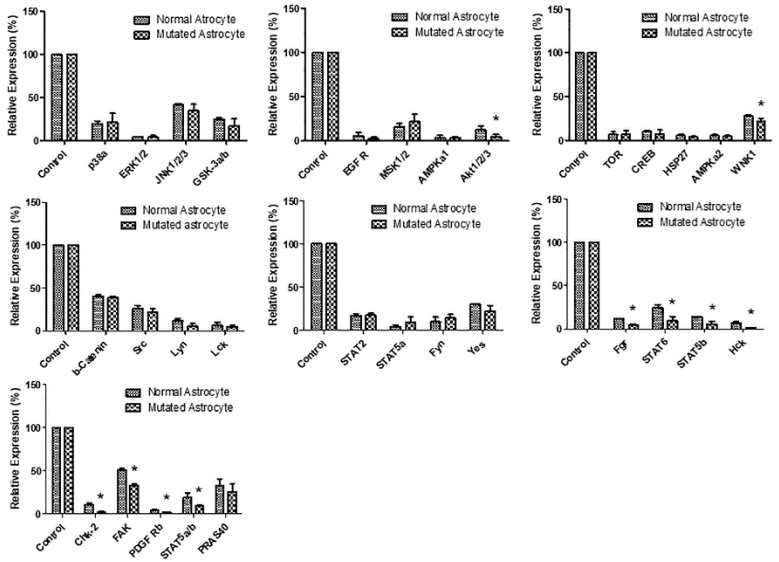

Figure 4: Normalised phospho-kinase expression of mutant astrocytes compared to a normal control. An asterisk (*) is displayed when the p- value was less than or equal to 0.05; N = 2.

Inflammatory Cytokines Detected in Microglia and Astrocytes

The mutated microglia and astrocytes, showed a total of 27 differentially expressed cytokines. Thirteen cytokines were significant ($p < 0.05$) between the mutated astrocytes and their control, whereas the remaining 14 cytokines were significant ($p < 0.05$) between the mutated microglia and their control (Table 1).

Response of Retinoic Acid Receptor and Melatonin to Mutations

We observed a tendency in the mutated cells to decrease retinoic acid receptor (RXR) alpha (α), beta (β), and gamma (γ). However, none of them exhibited a significant decrease in RXR α ($p = 0.98$), RXR β ($p = 0.89$), or RXR γ ($p = 0.95$) (Figure 5A and 5B). Melatonin was also non-significantly increased in the mutant microglia and astrocytes (Figure 5C).

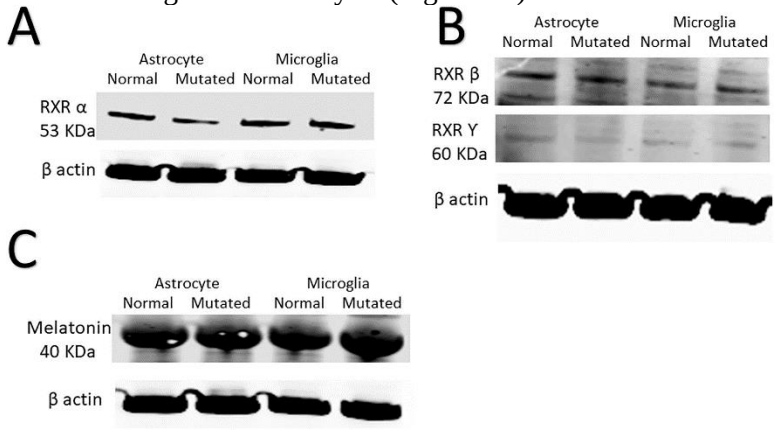

Figure 5: Immunoblots showing the expression of retinoic acid receptors (RXRs) and melatonin. (A) A western blot showing protein expression of RXR alpha (α) for mutant microglia and astrocytes compared to normal controls. (B) A western blot showing protein expression of RXR beta (β) and gamma (γ) in mutant microglia and astrocytes compared to normal controls. It is worth noting that RXR β and RXR γ were examined in the same blots. Therefore, the loading controls were reused for both entities. (C) A western blot showing the protein expression of melatonin for mutant microglia and astrocytes compared to normal controls; N = 3.

Table 1: Evaluation of cytokine expression in normal controls and in *CYP1B1* mutated cells.

	Cytokine Name	Expression in Normal Astrocytes N = 3	Expression in Mutated Astrocytes N = 3	Expression in Normal Microglia N = 3	Expression in Mutated Microglia N = 3
1	APRIL/TNFSF13 (42)	High	Low*	Not significant	Not significant
2	BAFF/TNFSF13B (37)	#	#	High	Low*
3	sCD30/TNFRSF8 (53)	Not detected	Not detected	#	#
4	sCD 163 (46)	Low	High*	#	#
5	Chitinase3-like1 (72)	High	Low#	Not detected	Not detected
6	gp130/sIL-6Rß (14)	High	Not detected	Not detected	High
7	IFN-a2 (20)	Not detected	High*	High	Low
8	IFN-ß (44)	#	#	Not significant	Not significant
9	IFN-g (21)	High	Low*	High	Low*
10	IL-2 (38)	Not significant	Not significant	High	Low*
11	SIL-6Ra (19)	High	Low*	#	#
12	IL-8 (54)	Not detected	Not detected	High	Not detected*
13	IL-10 (56)	Low	High*	Not significant	Not significant
14	IL-11 (39)	High	Not detected*	Not detected	Not detected
15	IL-12 (p40) (28)	Not significant	Not significant	High	Low*
16	IL-19 (29)	Not significant	Not significant	Not significant	Not significant
17	IL-20 (30)	Not detected	Not detected	Not detected	High*
18	IL-26 (22)	Not significant	Not significant	Not significant	Not significant
19	IL-27 (p28) (13)	#	#	Low	High*
20	IL-28A/IFN-Y2 (66)	High	Not detected*	Not significant	Not significant
21	IL-29/IFN-Y1 (33)	Not detected	Not detected	High	Not detected*
22	IL-32 (35)	Not detected	High*	Not detected	Not detected
23	IL-34 (15)	Not significant	Not significant	High	Low*
24	LIGHT/TNFSF14 (51)	High	Low*	High	Not detected*
25	MMP-1 (43)	Not detected	Not detected	#	#
26	MMP-2 (26)	Not detected	High*	High	Not detected*
27	Osteopontin(OPN) (77)	Not detected	High*	#	#
28	Pentraxin-3 (48)	Not detected	Not detected	High	Not detected*
29	sTNF-R1 (73)	Not detected	Not detected	Low	High*
30	sTNF-R2 (67)	High	Not detected*	High	Not detected*
31	TSLP (52)	Not significant	Not significant	Low	High*

'*' indicates significant results ($p < 0.05$). 'Not detected' indicates that the probe did not work or a negative result was reported.

'#' indicates that at least two probes out of four did not work, which means that the results probably need confirmation.

All data were obtained based on fixed standards supplemented in the kit. 'High' and 'low' labels were used based on the same standards. For concentrations, see S2 Table.

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Discussion

The *CYP1B1* gene is one of the major genes mutated in PCG. Abu-Amero et al. have demonstrated a reduced probability for *CYP1B1* mutation to cause primary open-angle glaucoma in heterozygous patients [23]. However, more convincing evidence for the mutated gene's contribution to primary congenital glaucoma PCG has been reported by Teixeira et al. which documented trabecular meshwork abnormalities in *CYP1B1* knockout mice (null mice) [24]. In addition, Safari et al. reported that the pG61E (p.Gly61Glu) mutation in the *CYP1B1* gene affects the extracellular matrix (ECM) in both humans and mice. ECM deficiency was documented in the human skin biopsies of two patients with a *CYP1B1* mutation [25]. The study suggested that the ECM damage was due to oxidative stress [26]. It is

known that ECM is produced and repaired by mesenchymal stem cells [27]. Likewise, mesenchymal stem cells secrete anti-inflammatory mediators to protect retinal ganglion cells [28].

Our study investigated the effects of *CYP1B1* mutation (p.Gly61Glu) on the primary astrocytes and microglia of rats in addition to human skin mesenchymal stem cells. We acknowledge that astrocytes and microglia isolated from rat brains may not be the same as cells isolated from the optic nerve head of rats. This limitation may contribute to invalid conclusions. Mutated astrocytes and microglia showed a 10% increase in proliferation in the first 24 hrs, (Figure 1A), which is most likely due to the activation of both cells. This increase, however, was not long lasting, and the increase in activity dropped by an average of 40% in 72 hrs (Figure 1B), suggesting that the activation was temporary. The decrease in proliferation at 72 hrs was statistically significant in microglia only (Figure 1B), suggesting that the *CYP1B1* mutation effect was more severe in the microglia. We should account for interference of the term's proliferation, cell growth and hyperactivity when interpreting metabolic activity data obtained at 24 hrs or more. Investigating oxidative stress in astrocytes and microglia revealed a reduction in the NADP/NADPH ratio in microglia only (Figure 2A and 2B). The reducing equivalent in the microglia which was declined by 20–45%, may contribute to the pathogenesis of reactive oxygen species (ROS). Skin mesenchymal stem cells (Hs27) play a major role in ECM repair and ROS inhibition [29]. Hs27 were tested under the stress of the p.Gly61Glu mutation in the *CYP1B1* gene and showed a 40% reduction in their ability to proliferate after 72 hrs (Figure 1C). This suggests potentially less repair to the ECM or ROS inhibition when damage to the environment occurs. Furthermore, it suggests less protection to retinal ganglion cells when progressive inflammation is present. Luckily, evaluating the NADP/NADPH ratio within *CYP1B1*-mutated Hs27 revealed no significant ROS abnormality compared to the control (Figure 2C). With the above results, we have supported the findings of other studies by confirming the existence of a potential leak of free radicals into the microenvironment. Additionally, the mutated microglia showed 71% pre-apoptosis, suggesting

programmed cell death within a few days (Figure 1D). High numbers of dead and activated microglia contribute to elevated inflammation. However, we suggest that microglia probably contribute to the ROS leak in PCG, among other factors.

We report upregulation of ERK1/2, GSK3a/b, EGFR, STAT5a and Hck cytokines in mutant microglia (Figure 3). It was previously discovered that ERK1/2 and EGFR promote a proinflammatory response [30]. In addition, GSK3a/b is a key protein known to regulate the balance between pro-inflammatory and anti-inflammatory responses [31]. Microglia proliferation is reduced when either STAT5 or HcK is inhibited [32]. The Hck gene is known to be upregulated in activated microglia [33]. Furthermore, we report down-regulation of Lyn, STAT6, STAT5b, focal adhesion kinase (FAK) and PDGF-Rb cytokines in mutant microglia. Microglia migration is controlled by Lyn [34]. In microglia, the clearance of dead cells, debris and the resolution of inflammation are promoted by STAT6. Similarly, cell adhesion, proliferation, migration, survival and pro-inflammation are mediated by FAK in microglia [35]. Microglia expressing PDGFRB have been found to promote vascular wall proliferation and repair [36].

In our study, the mutant astrocytes down-regulated the following phosphorylation proteins: akt1/2/3, WNK1, Fgr, STAT6, STAT5b, Hck, Chk2, FAK, PDGF Rb and STAT5a/b (Figure 4). The reduction of WNK1 is known to attenuate neural pain and reactive astrocytosis through NKCC1 inhibition [37]. Neural apoptosis is reportedly mediated by Hck [38]. Activation of akt1/2/3 and STAT6 in astrocytes promotes an anti-inflammatory response, as found in a previous study [39]. The damage response of DNA increases Chk2 expression in astrocytes, as found in a previous study [40].

In mutant astrocytes, we report upregulation of the following cytokines compared to the control: sCD63, IFN-a2, IL-10, IL-32, MMP-2 and osteopontin (Table 1). Out of these cytokines, IFN-a2, IL-32, sCD63 and osteopontin are pro-inflammatory, whereas IL-10 is the only anti-inflammatory cytokine upregulated [41]. IL-10's status as an anti-inflammatory cytokine

suggests activation of the feedback loop that is activated when inflammation progresses for a long period of time [42]. In addition, mutant astrocytes over-produce MMP-2, which is involved in ECM breakdown. This over-production probably increases the severity of ECM breakdown under inflammatory conditions.

In mutant microglia, we report upregulation of the following cytokines compared to the control: gp130, IL-20, IL-27 (p28) and TSLP (Table 1). These cytokines are involved in microglia activation and neural protection [43]. *CYP1B1* and retinoic acid receptors (RXR) contribute to the maintenance of the homeostasis of the following endogenous complexes: steroid hormones, fatty acids, melatonin and vitamins [44]. In addition, *CYP1B1* has been reported to be important for nervous system and vision health by interacting with and regulating melatonin metabolism. Melatonin is produced in the retina, lacrimal gland, lens and ciliary body of the eye [45]. On the other hand, melatonin has been reported to inhibit *CYP1B1* via a feedback loop [44]. The expression of RXR isoforms and melatonin (*MTNR1B*) in the context of astrocytes and microglia has been investigated. Decreased *CYP1B1* expression reduces fatty-acid oxidation, which involves RXR activities [44]. We report that RXR, RXR and RXR did not change in either mutated astrocytes or mutated microglia (Figure 5A and 5B). It seems that RXR isoforms did not respond to *CYP1B1* inhibition. In addition, no significant changes in melatonin were observed after mutating the *CYP1B1* gene (Figure 5C). This result was interesting because others have reported a link between *CYP1B1* expression and melatonin expression [46].

Melatonin was previously reported to stimulate neuronal cell survival involving the Akt/ NF-B pathway [47]. Most likely, melatonin homeostasis within astrocytes and microglia differs from the current understanding in the published research.

Based on the aforementioned data, we conclude that the p.Gly61Glu mutation within the *CYP1B1* gene induces microglial response. Additionally, the mutation reduced proliferation in all tested cell types. Furthermore, the mutation

tended to increase ROS and apoptosis, which contribute to damaging effects on the ECM and/or trabecular meshwork. Likewise, the increased production of MMp-2 further damages the ECM in the environment or tissue. Moreover, microglia are important for retinal ganglion cell survival because they play an important role in clearing pathogens and inflammation [48]. Severe *CYP1B1* mutation may damage eye development through loss of microglia, decreased ECM and increased ROS. We suggest more experimental testing to understand whether the identified progressive inflammation is preventable via non-invasive procedures, such as drugs. We also suggest investigating whether progressive inflammation is a cause for failure of glaucoma trabeculectomy, which is a surgery to remove eye-drainage tubes to allow fluid to drain smoothly.

Implications of CYP1B1 in Glaucoma and CancerCYP1B1

Cytochrome P450 (CYPs) are a group of enzymes consist of a large heme prosthetic group catalyzing the oxidation reactions of a wide range of organic substrates. P450 proteins were the first group of enzymes to be classified as a "superfamily" and the total number of enzymes belong to this family is more than 2100 members [49]. CYP1 is responsible for the phase I metabolism of endogenous and exogenous substrates and they are involved in the oxidative metabolism of endogenous materials, such as bile acids, steroid hormones, and lipids. Furthermore, CYP1 proteins metabolize potential carcinogens such as aryl hydrocarbons, aromatic amines, heterocyclic aromatic amines, and heterocyclic amines. CYP1 family has an essential role in procarcinogen activation, catalyzing the metabolism of ~ 66% of potential carcinogens. The biotransformation of procarcinogens causes the creation of mutagenic materials, which form adducts with nucleic bases, responsible for the beginning of carcinogenesis [50]. The cytochrome P450 CYP1 family consists of the CYP1A, CYP1B, CYP1C and CYP1D subfamilies [51-53]. Among the human P450 subfamilies, the CYP1B1 gene is the largest known in terms of mRNA size and number of amino acids; it is also the simplest in terms of its structure [51]. It is found on chromosome 2p21-22. Its length is approximately 8.5

kilobases (kb) of DNA, consisting of three exons and two introns. The size of its mRNA is 5.1 kb [54,55]. It has an open reading frame (ORF) starts at the 5' end of the second exon and encodes a predicted protein of 543 amino acids (**Figure 6**) [54]. In humans, CYP1B1 is expressed constitutively in healthy cells including eye, lungs, heart, kidney, prostate, brain, liver, uterus, ovaries and placenta [56-59]. It is also expressed in cells of the immune system and estrogen target tissues, such as mammary glands [59,60]. CYP1B1 expression in most organs was reported in the GTEx database as of October 21st, 2019 using GTEx Analysis version 7 (**Figure 7**) [61-63]. Tissues are known to accumulate melatonin or 6-hydroxymelatonin also has high expression levels of the CYP1B1 protein [64]. CYP1B1 is one of the most important enzymes involved in the metabolic activation of pre-carcinogens [11], such as polycyclic aromatic hydrocarbons [65], oxides, amines, and estrogens (**Figure 8A**) [66]. Moreover, CYP1B1 polymorphisms have been incriminated as risk factors in different types of cancers and glaucoma [67]. Here, we focus on the correlation between CYP1B1 and the development of glaucoma and cancer.

CYP1B1 in Glaucoma

Glaucoma is a heterogeneous disease that represents the second primary cause of irreversible loss of eyesight worldwide [68,69]. Globally, ~64.3 million individuals are diagnosed with glaucoma. This prevalence is anticipated to increase to 76 million in 2020 [70] and 111.8 million in 2040 [71]. Glaucoma is a nonuniform group of neurodegenerative ocular disorders [72] characterized by several clinical features that include visual field defects, retinal ganglion cell death, and progressive degeneration of the optic nerve [68]. The primary source of the progressive degeneration of the optic nerve is the occurrence of a lesion in the axons of the retinal ganglion cells [73]. International Classification (CGRN/WGA) of Childhood Glaucoma divided glaucoma into two types: Primary childhood glaucoma and Secondary childhood glaucoma. The primary childhood glaucoma is classified into Primary congenital glaucoma (PCG; OMIM 231300) and Juvenile open-angle glaucoma (JOAG; OMIM 137750). The Secondary childhood glaucoma is

classified into four categories: glaucoma associated with nonacquired ocular anomalies, glaucoma associated with nonacquired systemic anomalies, glaucoma associated with the acquired condition, and glaucoma following cataract surgery [74]. In children, the most common form is PCG, with a prevalence rate of 1:10,000 to 18,000 live births worldwide [75,76]. The frequency of CYP1B1 mutations in people with PCG depends on the population being considered. For example, in Saudi Arabian and Slovakian Roma residents, CYP1B1mutations were detected nearly in all cases of PCG [77]. Additionally, the prevalence of mutations in Brazil is 50%, in Indonesia it is 30%, and in Japan, it is 20%. Therefore, variability in prevalence indicates that other main genetic factors for PCG have not been identified yet. PCG is usually inherited as an autosomal recessive disease with incomplete penetrance [78]. This form is considered a rare form of glaucoma. It is characterised by isolated angle anomalies, possibly involve mild congenital iris anomalies, ocular hypertension, buphthalmos (enlargement of the eye), oedema, corneal opacification (scarring or clouding of the cornea), descemet's membrane herniation, lower density of the anterior sclera, iris atrophy and increased depth of the anterior chamber. Patients with PCG manifest variable symptoms include photophobia (intolerance to visual observation of light), blepharospasm (involuntary closing of the eyelids), and increased lacrimation (tear discharge) [78]. Optic neuropathy is not the sole contributor to PCG; glial cells are also considered major players in disease pathogenicity [79]. The development of ocular structures also involves the CYP1B1 enzyme [54], as confirmed by the detection of the CYP1B1 protein in several ocular cells of normal human fetal and adult eyes via immunolocalization assays. The intensity of CYP1B1 expression in fetal compared to adult human eyes indicates much more intense CYP1B1 immunolabeling. Anti-CYP1B1 immunoreactivity was evident in the primitive ciliary epithelium, non- pigmented ciliary epithelium, corneal epithelium, and keratocytes but was absent in the trabecular meshwork in all samples [58]. Notably, CYP1B1 was expressed in the human fetal nasal mucosa [80].

Although the diagnosis of PCG depends on clinical findings, the genetic tests could confirm the diagnosis. Several efforts have been initiated to understand the underlying mechanisms of the disease by using genome-wide association studies (GW AS) and genome sequencing technologies. Multiple PCG loci have been mapped; however, only *CYP1B1* has been identified to date as the leading cause of PCG. Interestingly, more than 150 mutations in *CYP1B1*, including missense, nonsense, regulatory, insertions, or deletions, have been associated with PCG [78]. In glaucoma, most scientists focus on the optic nerve. However, optic nerve supporting cells and their environments are not trivial. In glaucoma, characterized CYP1B1 mutations have not been properly investigated in astrocytes, microglia, oligodendrocytes, or other neural stromal cells. In PCG, patients usually have a global mutation that increases disease severity at various levels and in a variety of cells. Therefore, scientific awareness should be practiced in areas not covered entirely in the scientific literature, and hopefully, research will be geared towards them in the future.

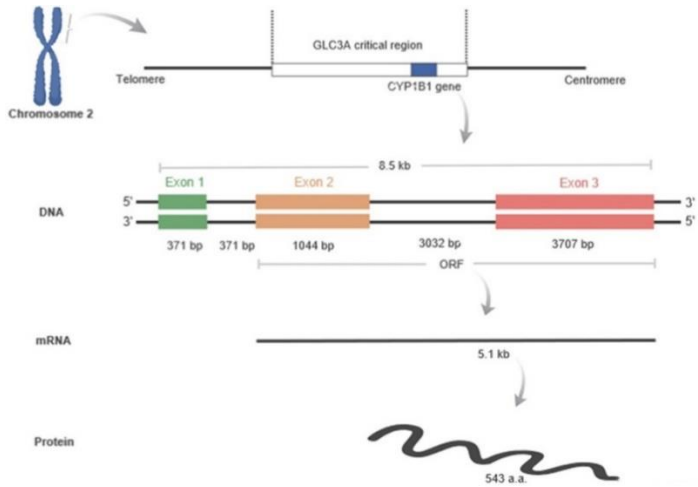

Figure 6: Human CYP1B1 located at 2p21-22 in the GLC3A critical region. The length of this gene is 8.5 kilobases, and it contains three exons and two introns: exon1 (371 bp), intron 1 (371 bp), exon 2 (1044 bp), intron 2 (3032 bp), and exon 3 (3707 bp). The open reading frame (ORF) starts from exon 2 and continues to exon 3. The ORF is translated into about 5.1 kb mRNA, which encodes a predicted 543 amino acids. Figure created by Biorender software.

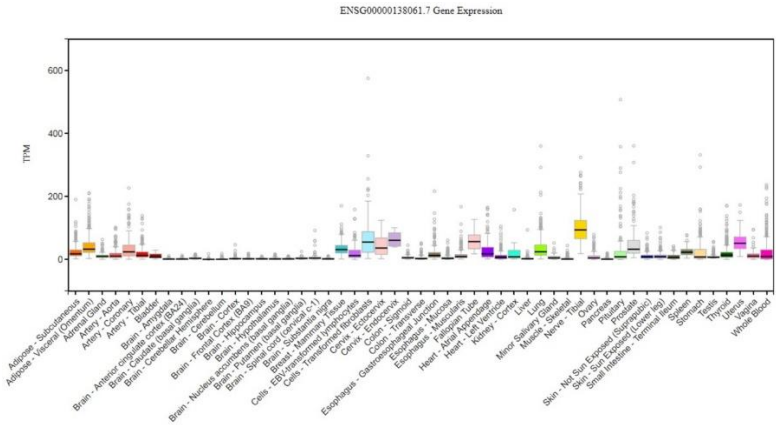

Figure 7: *CYP1B1* gene expression across diverse normal human tissues. The source of this Figure is Genotype-Tissue expression (GTEx) project database, gtexportal.org [61-63].

CYP1B1 in Cancer

CYP1B1 belongs to a group of enzymes known as Cytochrome P450s [51]. Interestingly, CYP1B1 is one of the main enzymes that are required in the hydroxylation of estrogens and the activation of some carcinogens. CYP1B1 mediates the metabolic activity of many procarcinogens. Polycyclic aromatic hydrocarbons (PAHs) are an example of a procarcinogen in pulmonary cells that can be converted to carcinogenic metabolites capable of producing DNA adducts (**Figure 8B**). For instance, quinones react with the N-7 of guanine and N-3 of adenine in DNA. This reaction generates DNA adducts that are capable of producing DNA mismatches during the DNA replication process, as well as modifying promoter methylation or promoter binding, leading to an inheritable DNA mutation or irregular gene expression, and eventually tumorigenesis. The reactive metabolites of PAHs may also motivate the formation of protein adducts in cells, which may influence the normal activities of these proteins. Additionally, PAH metabolites may also increase the production of reactive oxygen species (ROS), which can affect DNA, lipids, or proteins and commence carcinogenesis [81].

Furthermore, up-regulation of CYP1B1 is an indication of cancer, since it is highly selectively expressed in cancerous

rather than healthy tissues [82]. High CYP1B1 expression is found in multiple malignant tumors, including those of the brain, breast, colon, ovarian, and prostate cancers [51,83]. Immunohistochemical investigations have confirmed CYP1B1 overexpression in a majority of ovarian cancers. Most primary ovarian cancers (92%) show CYP1B1 immunoreactivity, present in the cytoplasm of the tumor cells. It is also detected in a majority (94%) of metastatic ovarian cancers. Some groups consider the CYP1B1 protein to represent an anticancer drug [84]. In a study done by Kwon and Chun on different cell lines, including MCF-7, MDA-MB-231, and HeLa cells, they reported that CYP1B1 increased invasion and inhibited cancer cell apoptosis [85]. Other studies have reported the effects of CYP1B1 knockout in vivo. When carcinogenic materials were delivered into mice with CYP1B1 knockout, decreased tumor growth, and increased DNA adduct formation was observed. These reports strongly suggest that CYP1B1 is a promising cancer biomarker and a potential target for the development of anticancer drugs [86]. However, a comprehensive molecular mechanism describing how CYP1B1 contributed to oncogenesis remains unknown [85].

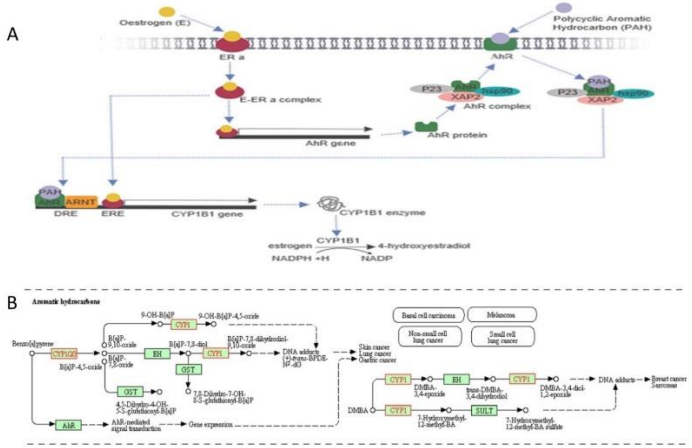

Figure 8: Aromatic hydrocarbon in conjunction with *CYP1B1* signalling pathway. **A,** Oestrogen binds with the oestrogen receptor alpha (ERa) to form an oestrogen-oestrogen receptor alpha complex (E-ERa), which induces an aromatic hydrocarbon receptor (AhR) gene. The AhR protein attaches to heat shock protein-90 (hsp90), XAP2 and P23 to form an AhR complex, then transport into the cell membrane. Also, AhR binds with a polycyclic aromatic

hydrocarbon (PAH) ligand to form an AhR complex/PAH complex. The AhR complex dissociates to form a heterodimer, AhR-ligand/ARNT. The heterodimer with oestrogen promotes the CYP1B1 gene. The CYP1B1 enzyme is involved in oestrogen metabolism by converting oestrogen into 4-hydroxyestradiol, which depends on NADPH. The Figure created by Biorender software. **B,** CYP1B1 gene involved in carcinogenesis pathway using Aromatic hydrocarbons leading to different types of cancers. The Figure obtained from KEGG pathways database.

Molecular Mechanism of CYP1B1

Wnt/β-catenin signaling may be associated with epithelial-mesenchymal transition (EMT) because they both need β-catenin [87]. To understand CYP1B1 molecular mechanisms, multiple studies have measured key oncogenic proteins including β-catenin, c-Myc, and ZEB-2 after CYP1B1 modulation. Jung Kwon et al. explored the role of CYP1B1 in cancer development and progression, trying to find out the leading molecular players that drive CYP1B1- mediated oncogenesis. β-catenin plays a vital role in mediating the Wnt/β-catenin signaling pathway. Upon the binding of the Wnt ligand to the receptor, β-catenin escapes the proteasomal degradation and trans locates into the nucleus, where it unites with its target genes and activates various signaling pathways involved in carcinogenesis. Furthermore, Jung Kwon and colleagues measured different markers of tumor progression, including cell proliferation, invasion, and cell migration after CYP1B1 modulation, induction, or inhibition. They observed multiple target proteins, including CTNNB1, ZEB2, SNAIL1, and TWIST1, that are correlated with EMT and Wnt/ β-catenin signaling were upregulated. Their findings indicate that CYP1B1 induces EMT and activates Wnt/β-catenin signaling via upregulating Sp1, a transcription factor that participates in cell proliferation and metastasis. This cascade of cellular actions inhibited E-cadherin expression and increased Wnt/β-catenin signaling upon the upregulation and nuclear translocation of β-catenin. Therefore, this was the first published study that reported the molecular mechanisms of how CYP1B mediates tumorigenesis [85]. A more detailed understanding of the underlining mechanisms of CYP1B1-mediated carcinogenesis may help in the development of new methods for cancer treatment.

Sex hormone metabolism has defected in mammary gland tumorigenesis, and variable metabolites may have different effects on mammary epithelial cells. 4-hydroxy estradiol (4-OHE2), an oxidized metabolite of estrogen, is significantly expressed in human breast cancer cells. This catechol estrogen has been proposed as an inducer of hormonal carcinogenesis, such as breast cancer. Furthermore, when transgenic mice with increased expression of CYP1B1, an essential enzyme of 4-hydroxy metabolites [11], were stimulated with estrogen, cancer cells were found in their mammary glands [88].

Hypomethylation in the promoter region of CYP1B1 regulates the overexpression of this gene, as determined in clinical prostate tissues and prostate cancer cell lines using methylation-specific PCR and bisulfite-modified DNA sequencing [89]. Another study investigated the function of miRNA in drug-metabolizing enzymes provided another reason for elevated CYP1B1 levels. The results of luciferase assays suggested that miR-27b regulates CYP1B1 at the post-transcriptional stage. A decreased level of miR-27b was associated with elevated levels of CYP1B1 protein in cancerous tissues [90]. Recently, another miRNA, miR-187-5p, was also shown to have decreased expression in non-small lung cancer. Interestingly, CYP1B1 is a direct target gene for miR-187-5p, which is expressed during cancer cell growth [91].

Moreover, elevated levels of the pro-inflammatory cytokine interleukin-6 also have effects on CYP1B1 expression in HCT116 and SW480 colorectal cancer cell lines. This cytokine can manipulate CYP1B1 expression and can induce the activation of dietary carcinogens and DNA damage that can trigger colorectal cancer progression [92]. Additionally, the breast cancer microenvironment can be guided by leptin, which directly stimulates estrogen metabolism genes, including CYP1B1, that are involved in the oxidative metabolism (4-hydroxylation) of estrogen. Oxidative metabolism of estrogen leads to DNA adduct formation and cancer mutations [93].

Cigarette smoke carcinogens such as 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and polycyclic aromatic hydrocarbons (PAH) are known as significant causes of lung cancer, which kills on average 3000 people per day worldwide [94]. NNK and PAH upregulate CYP1B1 enzyme activity upon

binding to AhR (**Figure 8B**). The AhR overexpression upregulated the expression of CYP1B1 in the early stage of lung adenocarcinoma, which could increase intracellular oxidative stress and promote cell growth. Therefore, the disruption of AhR expression could prevent the early development of lung adenocarcinomas [95].

Flavonoids are a group of common substances with variable phenolic structures. These compounds are found in natural products such as fruits, vegetables, grains, blossoms, tea, and wine. These particular items are well known for their beneficial impacts on wellbeing, and tremendous efforts are being made to isolate Flavonoids from these natural products. Because of Flavonoids anti-oxidative, anti- inflammatory, and anti-carcinogenic properties and due to their ability to balance main cellular enzyme functions, they are considered as an essential component in multiple nutraceuticals, pharmaceutical, medicinal, and cosmetic uses [96]. Flavonoids have a tight, rigid structure that enables them to fit precisely within the active site of the CYP1B1 enzyme [66]. Interestingly, dietary flavonoids can be metabolized by CYP1B1 to products that can inhibit cancer proliferation [97]. Furthermore, many studies recommended the use of flavonoids to treat several types of cancer, such as melanoma, hepatocellular carcinoma, and thyroid cancer. The administration of flavonoid in animal models of cancer has enhanced the tolerance to cancer treatment [96]. In cancer cells, CYP1B1 is highly expressed. This observation proposes that CYP1B1 enzyme may metabolize variable anticancer drugs. Cisplatin is considered an essential chemotherapeutic agent, but it might also play a role in chemo-resistance. The presence of CYP1B1 in tumor cells decreases the sensitivity of the cells to the cytotoxic effects of Cisplatin. However, the co-administration of potent, cell permeable and water soluble CYP1B1 inhibitors, and 6j (DMU2139), with Cisplatin can overcome CYP1B1-mediated resistance and re-sensitized cancerous cells once again to the Cisplatin [98]. These findings suggest the useful roles of CYP1B1 inhibitors in the treatment of cancer.

Prognosis and Interactions of CYP1B Gene

Looking at TCGA database, significance was found for CYP1B1 expression relevant to bladder and kidney carcinoma. Low expression of CYP1B1 in cancer corresponded to better survival detected by Kaplan-meier plot for kidney (renal) clear cell carcinoma and bladder urothelial carcinoma with p-value 0.00265 and 0.0175 respectively (Figure 9). The data was extracted from over 200 patients suffering from cancer in both diseases [99]. This finding opens the door for scientists to investigate possible treatment methods such as CYP inhibitors to achieve better survival in both cancers.

Interaction of CYP1B1 genes have been documented in many research papers through STRING database. Level of confidence was assessed based on physical experiment, bioinformatics predictions, co-expressions anticipation and indirect association, See Figure 10. These interactions involve biological processes, molecular functions, and/or cellular components. The STRING database has identified 100 publications for CYP1B1 interactions and minimum 22 pathways when scale of confidence was set at high confidence. This information will enhance the scientific investigation toward validating predicted phenomenon or interaction. In addition, Table 1. lists functions of molecules that directly interact with CYP1B1.

Interestingly, dietary flavonoids can be metabolized by CYP1B1 to products that can inhibit cancer proliferation [97] Furthermore, many studies recommended the use of flavonoids to treat several types of cancer, such as melanoma, hepatocellular carcinoma, and thyroid cancer. The administration of flavonoid in animal models of cancer has enhanced the tolerance to cancer treatment [96]. Natural flavonoids are the primary source of CYP1B1 inhibitors. Methoxy types of flavones and flavonols were appeared to prevent CYP1B1 activity, such as chrysoeriol and isorhamnetin specifically. Various flavonoids including quercetin, rutin, apigenin, and amentoflavone, show an inhibitory effect of CYP1B1. Some CYP1B1 inhibitors, such as kaempferol and isorhamnetin, can also antagonize the expression of AHR, which may show synergetic inhibition on the expression of CYP1B1.

Interestingly, some anti-cancer drugs used in clinics are competitive inhibitors of CYP1B1, such as flutamide, paclitaxel, mitoxantrone, and docetaxel. CYP1B1 inhibitors can be utilized to understand the CYP1B1 function; thus, it might be considered as anti-cancer agents for the treatment of certain types of cancer [44]. In cancer cells, CYP1B1 is highly expressed. This observation proposes that CYP1B1 enzyme may metabolize variable anticancer drugs. Cisplatin is considered an essential chemotherapeutic agent, might also play a role in chemo-resistance. The presence of CYP1B1 in tumor cells decreases the sensitivity of the cells to the cytotoxic effects of Cisplatin. However, the co-administration of potent, cell permeable and water soluble CYP1B1 inhibitors, such as the synthetic pyridylchalcones 7k (DMU2105) and 6j (DMU2139), with Cisplatin can overcome CYP1B1-mediated resistance and re-sensitized cancerous cells once again to the Cisplatin [98]. These findings suggest the useful roles of CYP1B1 inhibitors in the treatment of cancer.

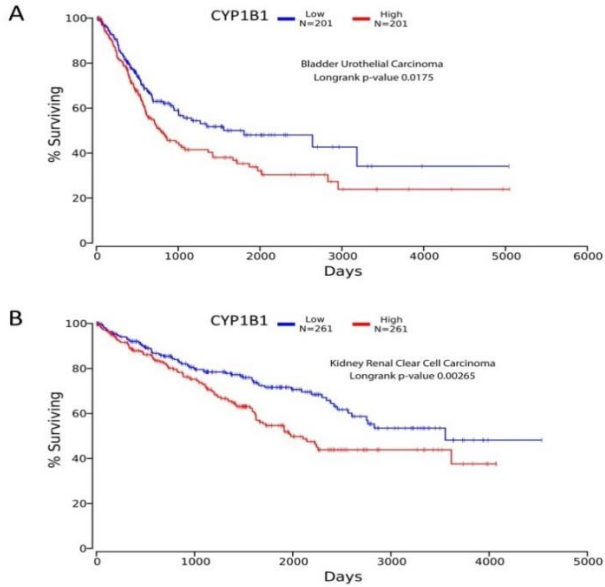

Figure 9: TCGA survival curves using Kaplan meier analysis. **A,** represents bladder urothelial carcinoma showing longer survival for low expression CYP1B1 patients. **B,** represents renal clear cell carcinoma showing extended survival for decreased expression CYP1B1 patients. Figures created from oncolnc.org website with TCGA database incorporated.

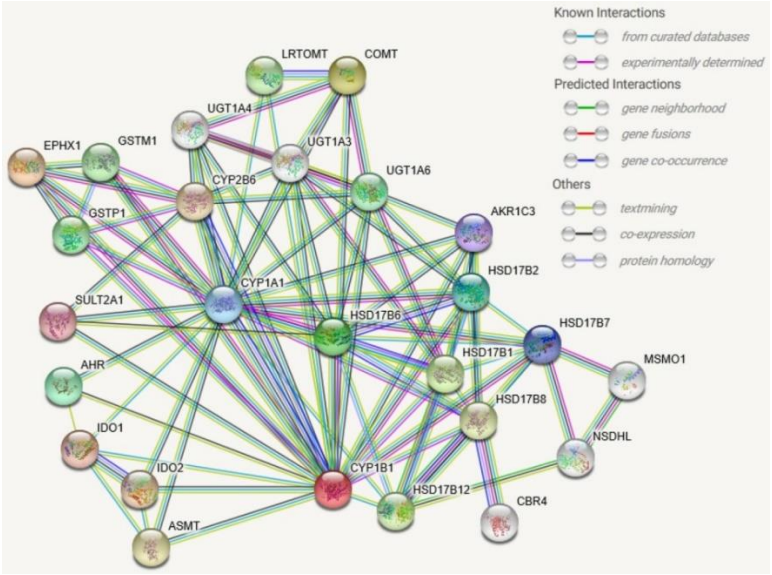

Figure 10: CYP1B1 protein-protein associations frpm STRING database. Colored nodes are query proteins and first shell of interactors. White nodes are second shell of interactors. filled nodes (structure inside the node) represent some 3D structure is known or predicted. Interactions include: i) known interactions from curated databases and experimentally determined, ii) predicted interactions by gene neighborhood, gene fusion, and gene co-occurrence, iii) others by text mining, co-expression, and protein homology. All interactions have been segregated by different colors.

Conclusion

High-advanced technologies, including whole- exome sequencing (WES) and whole-genome sequencing (WGS), helped to reveal the molecular mechanisms of how genetic abnormalities such as CYP1B1 mutations lead to glaucoma and cancer. Glaucoma-related abnormalities are severe and can cause irreversible blindness. The CYP1B1 gene is the most common gene mutated in PCG. Although many articles have pointed out the importance of this gene, molecular and physiological information are not completed yet. Due to the lack of research support, many mutations have been characterized, but others have been neglected. Also, some African and Middle-Eastern mutations are not characterized yet.

Furthermore, scientists are investigating either CYP1B1 mutations or CYP1B1 overexpression since they contribute to glaucoma or cancer, respectively. On the other hand, a balanced expression of CYP1B1 in either glaucoma or cancer has not been investigated. Thus, multiple questions remain unanswered, such as: does high expression in the eye cause cancer? Or does a reduction of expression in other organs, such as the liver, create difficulty? We have promising disease candidates for CYP1B1 inhibitor trials which are kidney renal clear cell carcinoma and bladder urothelial carcinoma. Additional information is needed to evaluate different treatment plans in those scenarios. With the development of new genetic engineering techniques, a possible option of treatment is the correction of the CYP1B1 abnormality via gene-editing technologies such as the CRISPR-Cas9 system.

Additionally, genome-wide association studies (GWAS) and other cellular techniques studying gene expression and regulation will undoubtedly change our understanding of how CYP1B1 mutations contribute to the pathogenesis of glaucoma and cancer. In order to achieve this, in the future, it is essential to have full access to glaucoma and cancer patient's samples. Also, it is vital to have an available platform occupied with all instrumental technologies needed to perform different research studies such as functional genomics, gene expression studies, pathway analysis, and epigenetic studies. Therefore, the ultimate knowledge we will get out from these studies will expand our understanding of the different cellular and molecular mechanisms of glaucoma and cancer, which in turn will help in the development of new therapeutic options for glaucoma and cancer patients.

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