Decreased expression of gap junction delta-2 (*GJD2*) messenger RNA and connexin 36 protein in form-deprivation myopia of guinea pigs

Guo-Yuan Yang[1], Feng-Yang Liu[1,2], Xia Li[1], Qiu-Rong Zhu[1], Bing-Jie Chen[1], Long-Qian Liu[1]

[1]Department of Opthalmology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China;
[2]Department of Ophthalmology, First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China.

Abstract

Background: More than ten genome-wide association studies have identified the significant association between the gap junction delta-2 (*GJD2*) gene and myopia. However, no functional studies have been performed to confirm that this gene is correlated with myopia. This study aimed to observe how this gene changed in mRNA and protein level in the form-deprivation myopia (FDM) animal model.

Methods: Four-week-old guinea pigs were randomly divided into two groups: control and FDM groups ($n = 12$ for each group). The right eyes of the FDM group were covered with opaque hemispherical plastic lenses for 3 weeks. For all the animals, refractive status, axial length (AL), and corneal radius of curvature were measured at baseline and 3 weeks later by streak retinoscope, A-scan ultrasonography, and keratometer, respectively. Retinal *GJD2* mRNA expression and connexin 36 (Cx36) levels in FDM and control groups were measured by quantitative real-time PCR and Western blot analyses, respectively. Those results were compared using independent t test, Mann-Whitney U test, or paired t test. A significance level of $P < 0.05$ was used.

Results: Three weeks later, the FDM group (form-deprived eyes) showed about a myopic shift of approximately -6.75 (-7.94 to -6.31) D, while the control group remained hyperopic with only a shift of -0.50 (-0.75 to 0.25) D ($Z = -3.38$, $P < 0.01$). The AL increased by 0.74 (0.61–0.76) and 0.10 (0.05–0.21) mm in FDM and control groups, respectively ($Z = -3.37$, $P < 0.01$). The relative mRNA expression of *GJD2* in the FDM group decreased 31.58% more than the control group ($t = 11.44$, $P < 0.01$). The relative protein expression of CX36 on the retina was lowered by 37.72% in form-deprivation eyes as compared to the controls ($t = 17.74$, $P < 0.01$).

Conclusion: Both the mRNA expression of *GJD2* and Cx36 protein amount were significantly decreased in the retina of FDM guinea pigs. This indicates that Cx36 is involved in FDM development, providing compensating evidence for the results obtained from genome-wide association studies.

Keywords: Connexin 36; Myopia; Guinea pigs; Gap junction

Introduction

Over the last three decades, the increasing prevalence of myopia has become an important health issue worldwide.[1,2] In some urban areas of southeast Asian countries, it affects 80% to 90% of adolescents.[3,4] Among myopes, about 10% to 20% cases develop into high myopia, which would cause irreversible vision loss complications, such as retinal detachment, macular retinoschisis, and myopic choroidal neovascularization.[5,6] The etiology of myopia is multi-factorial.[7] Evidence from animal experiments and epidemiology suggest that both environmental and genetic factors contribute to the genesis of myopia.

Multiple genome-wide association studies (GWAS) based on many ethnic groups have successfully identified numerous susceptible genes for myopia.[8] Among them, gap junction delta-2 (*GJD2*), which encodes the connexin 36 (Cx36) proteins, is one of the most promising genes. The association between *GJD2* and myopia was first identified in 2010 by Solouki *et al*.[9] More than ten independent replication studies and GWAS (including meta-GWAS) subsequently confirmed the association between *GJD2* and myopia.[10-19] As mentioned before, Cx36 is encoded by the *GJD2* gene. This connexin protein is widely expressed in cones, AII amacrine cells, bipolar cells, and ganglion cells on the retina in mammalian animals.[20-24] More importantly, Cx36 forms gap junction channels between adjacent membranes of photoreceptor cells.[23] Also, Cx36 is an essential protein in electrical synaptic transmission, which contributes to signal averaging and noise reduction in vision

processing.[25] All of the information above intrigued our interests regarding the relationship between Cx36 and myopia genesis. Therefore this study established a form-deprivation myopia (FDM) model in guinea pigs and investigated the changes in the expression of *GJD2* and Cx36 in the retina.

Methods

Ethical approval

Experimental procedures and animal treatments were conducted in accordance to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the whole protocol was approved by the Animal Care and Use Committee of the Sichuan University (No. 2016038A).

Animal model

Four-week-old guinea pigs were obtained from the Animal Laboratory Center of Sichuan University, China. Because our pilot study showed form-deprivation could induce significant myopic shift in guinea pigs, by calculating the sample size and power, a total of 24 guinea pigs were randomly divided into FDM ($n = 12$) and control group ($n = 12$). Guinea pigs were housed together in groups of three at 25°C on 12/12 h of light (500 lux)/darkness, with food and water available *ad libitum*. Special spectacle lenses used to adjust the eye size of guinea pigs were available from King Tak & Jia Run Company (King Tak & Jia Run Co., Ltd, Beijing, China). The lenses were made from polymethyl methacrylate materials with zero diopter (confirmed by lensometer). The parameters were as follows: overall diameter, 17.00 mm; optical diameter: 11.50 mm; inside optical curve radius: 7.50 mm; and outside optical curve radius: 7.64 mm. Lenses were steadily fixated to the skin around the eye of the guinea pigs by glue. The optical area was heavily scratched in the central parts of the spectacle lens in order to cause visual form deprivation. Guinea pigs in the FMD group wore scratched and dimmed lenses on the right eye the entire day, while their counterparts in the control group wore transparent plano lenses on the right eye. The left eyes were untreated in both groups. We checked the animals three times each day to make sure the lenses were correctly worn on the right eye.

Ocular biometry assessment

Ocular biometry assessments were performed before the experiment began and after myopia was induced, including refractive status (evaluated by spherical equivalent [SE]), corneal radius of curvature, and axial length. All of the biometric measurements were performed independently by two optometrists.

The refractive status was measured using a streak retinoscope with trial lenses in a dark room. For full mydriasis and cycloplegia, one drop of 0.50% compound tropicamide eyedrops (Santen Pharmaceutical Co., Ltd. Shiga Plant, Japan) was topically administered to the eye every 5 min for a total of four times. Retinoscopy was performed 30 min after the last drop. Refractions were recorded as SEs (spherical degree + 0.5 × cylindrical degree).

The axial dimensions were determined using A-scan ultra-sonography (AVISO Echograph Class I-Type Bat; Quantel Medical, Clermont-Ferrand, France) with a 10-MHz transducer. Topical corneal anesthesia was administered 0.40% oxybuprocaine hydrochloride (Santen Pharmaceutical Co., Ltd. Shiga Plant) before measurement. The ultrasound probe was in slight contact with the central part of cornea, with no pressure on it during the measuring process. The axial length of the eye was defined as the distance from the surface of the cornea to the internal limiting membrane. An average of ten measurements was recorded for analysis. The corneal curvature radius was measured by a portable automatic keratometer (Tianjin Suowei Electronic Technology Co., Ltd, Tianjin, China). The curvature radius of both the long and short axis of the cornea was measured when there were four clear and steady red dots simultaneously presented to the cornea. Eight readings were recorded for each measurement to provide a mean value.

Tissue extraction

After 21 days of experiments, all of the guinea pigs were deeply anesthetized with an intra-peritoneal injection of 10% chloral hydrate (200 mg/kg; Boster Biological Technology Co., Ltd., Wuhan, China). Eyes were quickly enucleated and excessive orbital fat was removed. After that, the eyes were cut into anterior and posterior segments at the limbus. After removal of the lens and vitreous, the whole retina was peeled carefully without the retinal pigment epithelium attached and promptly put into the Eppendorf tubes at −80°C until use.

Real-time reverse transcription quantitative polymerase chain reaction (qRT-PCR)

The total RNA from the retina in form-deprivation eyes and control eyes were separately isolated using TRIzol reagent (Invitrogen Co., Ltd, Carlsbad, CA, USA). Reverse transcription was performed using a Gold Scriptc DNA kit (Invitrogen Co., Ltd) according to the manufacturer's protocol. This study used Oligo 6 software (DBA Oligo, Inc. Colorado Springs, CO, USA) to design primers for *GJD2* and the β-actin gene that served as an internal reference. The primer sequences are listed in Table 1. A Q-PCR kit (Takara BioCo., Ltd, Beijing, China) with SYBR Green was used with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The total PCR reaction volume was 20 μL, and the reaction conditions were as follows: an initial denaturing phase of 95°C for 35 s; 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 35 s, and extension at 72°C for 40 s. After q-PCR, the relative quantification of *GJD2* expression was calculated by the $2^{-\triangle\triangle Ct}$ method, and the β-actin gene served as an internal control.

Western blotting analysis

First, the protein from retina tissues on each eye was extracted using radio-immunoprecipitation assay lysis

Table 1: Primers sequence for the real-time polymerase chain reaction.

Gene	Primer sequence	Length (bp)
GJD2	Forward: 5′CAGAGCCAGATTGTTTAGAAG3′	186
	Reverse: 5′GGGACACTGAAGCCATAGAG3′	
β-actin	Forward: 5′TTCTAGGCGGACTGTTACTAC3′	87
	Reverse: 5′CAATCTCATCTCGTTTTCTG3′	

GJD2: Gap junction delta-2.

Table 2: Biometric parameter comparison between FDM ($n = 12$) and control group ($n = 12$) at 0 and 3 weeks.

Parameters	FDM	Control	Statistics	P
0 week				
SE (D)	4.03 ± 1.45	3.94 ± 0.89	0.16[*]	0.88
AXL (mm)	7.86 ± 0.17	7.86 ± 0.17	0[*]	1.00
CC (mm)	3.37 ± 0.12	3.48 ± 0.15	−1.55[*]	0.14
3 weeks				
SE (D)	−2.03 ± 0.49	3.75 ± 1.15	11.42[*]	<0.01
AXL (mm)	8.49 ± 0.22	7.99 ± 0.22	4.53[*]	<0.01
CC (mm)	3.48 ± 0.15	3.43 ± 0.10	0.82[*]	0.82
C (0–3)				
SE (D)	−6.75 (−7.94 to −6.31)	−0.50 (−0.75 to 0.25)	−3.38[†]	<0.01
AXL (mm)	0.74 (0.61–0.76)	0.10 (0.05–0.21)	−3.37[†]	<0.01
CC (mm)	0.16 (0.06–0.23)	0 (−0.26–0.15)	−1.92[†]	0.06

Data were presented by mean ± standard deviation or median (interquartile range). [*]Student t test or [†]Mann-Whitney U test are used to analyze variables. FDM: Form-deprivation myopia; SE: Spherical equivalence; AXL: Axial length; CC: Cornea curvature; 0 week: Before experiment start; 3 weeks: At the end of experiment; C (0–3): Change of biometric parameters from baseline to the end of the third week.

buffer and quantified by using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Shanghai, China). All of the samples were boiled with 5× loading buffer (Beyotime Institute of Biotechnology) at 100°C for 5 min, and then 30 μg homogenated protein from each retina was loaded for sodium dodecyl sulfate-polyacrylamide gel, electrophoresed, and transferred to polyvinylidene fluoride membrane. The primary antibodies used in this study were as follows: rabbit against-Cx36 polyclonal antibody (LC-C101642; Lifespan, Seattle, WA, USA) at 1:1000, and β-actin (Abcam, ab6276, Cambridge, UK, used as a loading control) at 1:2000 dilution. After washing with Tris-buffered saline containing 0.1% Tween-20 (TBST) three times (15 min each time), the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit (1:2000; BA1054), and then goat anti-mouse (1:2000; BA1050, Boster Biological Technology Co., Ltd) IgG secondary antibodies for 1 h at room temperature. The membranes were subsequently washed three times (15 min each time) with TBST. Films were scanned by an Odyssey Western blot platform (LI-COR Biotechnology, Nebraska, USA). The optical densities of the immunostained bands on the film were processed by Alpha Ease FCTM software (Version 3.1.2, AlphaInnotech Corp., San Leandro, CA, USA). Each experiment was repeated three times.

Statistical analysis

As only 12 guinea pigs were included in FDM and control group, the normal distribution test was conducted in the variables by Kolmogorov-Smirnov test (SPSS version 20, Chicago, USA). The biometric parameters of SE, axial length, corneal curvature, mRNA expression, and Western blot protein are presented as the mean ± standard deviation or median (interquartile range). Measurements of the right eye in the FDM and control group were compared by independent t test or Mann-Whitney U test (SPSS version 20). Comparison of the relative GJD2 mRNA level and Cx36 amount in the right eye retina in FDM and control group were also conducted by paired t test. A significant level of $P < 0.05$ was used.

Results

Optic and biometric parameters

The results indicated that the variable of biometric parameters fitted normal distribution by Kolmogorov-Smirnov test. Before the start of experiment (0-week, at baseline), statistical analysis showed no significant difference between the FDM and control groups in SE, axial length, or cornea curvature. At 0-week, both the FDM and control group were hyperopic. After 3 weeks of form deprivation (3 weeks), the refractive status of eyes in the FDM group shifted from 4.03 ± 1.45 D to −2.03 ± 0.49 D. Form deprivation induced a myopic shift of approximately −6.75 (−7.94 to −6.31) D. Additionally, at the 3-week time point, eyes in the control group remained hyperopic with only a −0.50 (−0.75 to 0.25) D shift, which indicated that form deprivation induced many myopic changes in SE ($Z = −3.38$, $P < 0.01$). Results of the axial
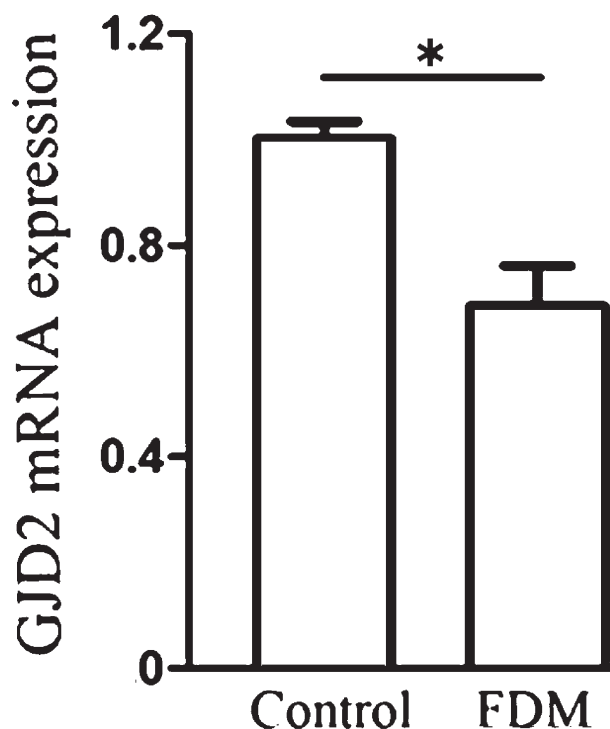
Figure 1: Relative mRNA expression of Cx36 in FDM and control groups at 3 weeks ($n = 12$). $^*P < 0.01$. Cx36: Connexin 36; FDM: Form-deprivation myopia group.

length coincided with SE. The mean increment of the axial length of 0.74 (0.61– 0.76) mm of axial length was found in FDM group, while their counterparts only increased 0.10 (0.05–0.21) mm ($Z = -3.37$, $P < 0.01$). However, the difference of corneal curvature between the 0 and 3-week only changed by 0.16 (0.06–0.23) and 0 (−0.26–0.15) mm in the FDM and control groups, respectively. No significant difference in corneal curvature was identified ($Z = -1.92$, $P = 0.06$). In conclusion, statistical analysis confirmed the success of the FDM model in guinea pigs with significant myopic shift and longer axial length [Table 2].

Relative mRNA and protein expression change of Cx36

After 3 weeks of form deprivation, the relative mRNA retinal expression of GJD2 in FDM eyes decreased 31.58% more than the control group after calibration with internal parameters ($t = 11.44$, $P < 0.01$) [Figure 1]. Congruously, at 3 weeks, the protein expression of Cx36 on the retina decreased by 37.72% in form-deprivation eyes as compared to the controls ($t = 17.74$, $P < 0.01$) [Figure 2].

Discussion

As mentioned above, genetic study identified *GJD2* as one of most susceptible gene for myopia. However, the role of *GJD2* and related potential pathway in myopia genesis was still unknown. This study observed the change in mRNA (GJD2) and protein (Cx36) expression in a FDM model. Although some studies have investigated the

immunocytochemical localization or function of Cx36 in guinea pigs or mice,[26-28] seldom study investigate GJD2 expression at the mRNA and protein level in a FDM model. We observed that after 3 weeks of form deprivation by wearing opaque lenses, the guinea pigs had obvious shifts toward myopia as compared to the controls. Previously, we created FDM models by covering guinea pig eyes with face masks modified with latex balloons.[29] Comparing the two methods, we found that wearing opaque lenses has a higher efficiency in creating an FDM model with lower SE. Also, a significant decrease of GJD2 mRNA and Cx36 protein expression on retinal tissue was observed compared to the control eyes. This further indicates that Cx36 was involved in the unknown pathway in myopia genesis. Previous study on Cx36 mainly focused on how it acts as an intercellular channel and increases the loss of neurons and optic nerve axons in a mouse model of glaucoma,[28] acts in the electrical coupling between mouse rods and cones,[30] and finishes sophisticated cell-cell communication networks via gap junction channels.[31] As little study has investigated the association between Cx36 and myopia or refractive development, we cannot compare our results to others directly. It is well-known that genetic study (especially GWAS) just finds susceptible genes for one disease. The gap between susceptible gene and causative gene should be filled by further function study. By confirming the CX36 change in mRNA and protein level on retina in FDM model, it provides more confidence on re-evaluating and highlighting gap junction on refractive development and myopia for us. Kiefer *et al*[18] classified the susceptible genes found by GWAS according to their possible connection with myopia, including extra-cellular matrix remodeling, the visual cycle, eye and body growth, retinal neuron development, and general neuronal development or signaling. After reviewing the function and relation of agents susceptible to myopia, it is considered that *GJD2* may contribute to myopia genesis by affecting neuronal signaling on the retina.

Cx36, a prominent gap junction protein on the retina, is widely expressed in cones, AII amacrine cells, bipolar cells, and photoreceptor cells.[32] First, electrical transmission network via gap junction is an important mode of inter-neuronal communication besides chemical synaptic trans-mission in the retina.[25,26,30,31] Electrical coupling between AII amacrine cells mediated by gap junctions results in signal averaging and noise reduction, and this process can be modulated by the intensity of background light.[25] Since emmetropization is driven by a high-quality retinal image, any abnormality in the retinal pathways could potentially interrupt normal refractive development. Second, an imbalance of the ON and OFF signaling pathway caused by the dysfunction of Cx36 may affect refractive development. In normal visual processing, the ON pathway supplies cross-inhibition to the OFF pathway to improve the efficiency of contrast encoding.[33] Electroretinogram recordings of Cx36 knockout retina under either dark-adapted or light-adapted conditions identified that the deletion of Cx36 partially disrupts the ON pathway. Recent research has revealed that a defective ON pathway increases susceptibility to FDM.[34] Third, the activity of Cx36 (as determined by its phosphorylation
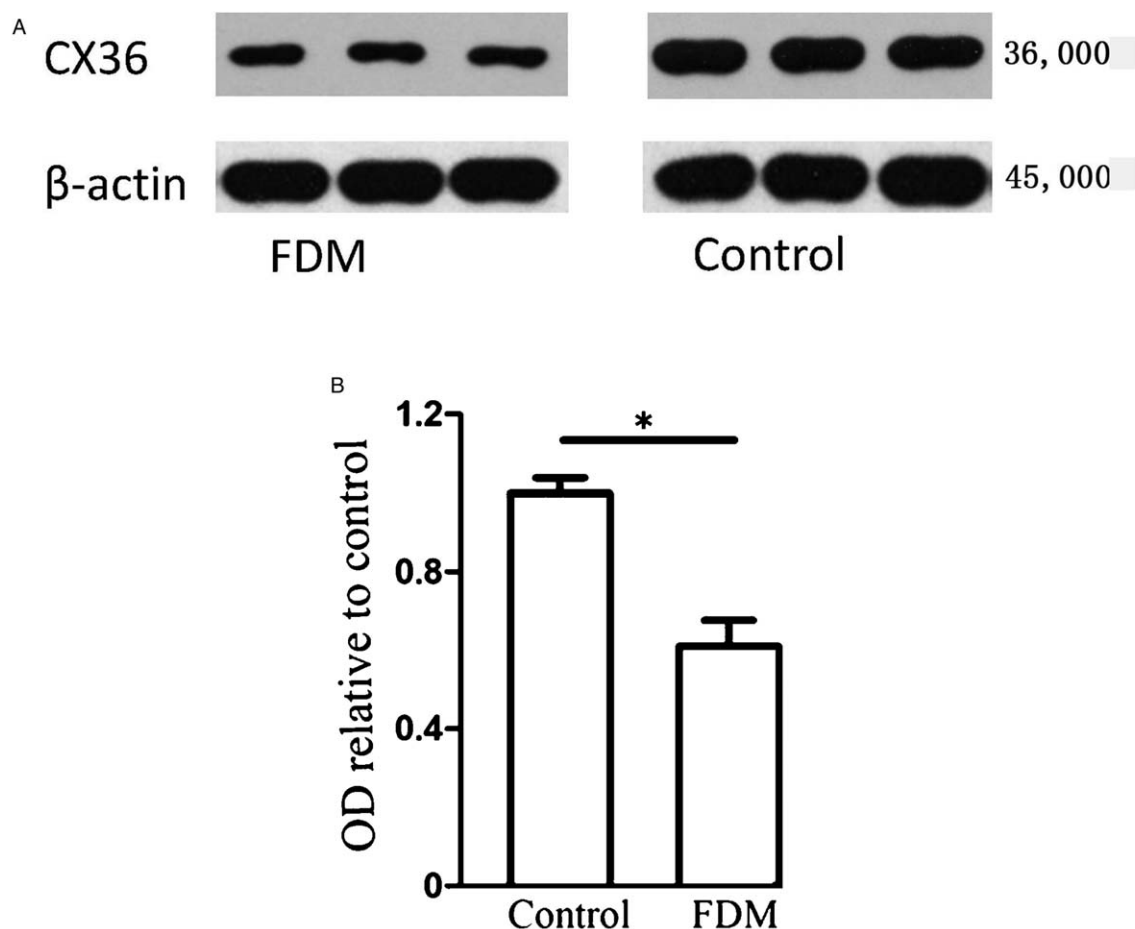
Figure 2: Western blotting test image of retina in FDM and control eyes at 3 weeks (A). Western blotting analysis of Cx36 expression on the retina in FDM and control eyes at 3 weeks (B; $n = 12$). $^*P < 0.01$. Cx36: Connexin 36; FDM: Form-deprivation myopia group; OD: Optic density.

level) appears to be regulated by light exposure and dopamine level, two widely accepted elements in eye growth and myopia control.[35] In addition, previous studies have identified that adenosine also regulates photoreceptor coupling by regulating the phosphorylation state of Cx36 in the retina of mice and zebrafish.[35,36] The cyclic adenosine monophosphate level modulates scleral collagen remodeling, which is a critical step in the development of myopia.[37]

This study observed a significant reduction of *GJD2* gene mRNA and Cx36 protein expression in a FDM model using guinea pigs. Combing with the results of previous genome-wide association studies on *GJD2* gene and immunology studies of CX36 protein on retina, we think that the decreased expression of mRNA and protein of *GJD2* did identified the relationship between Cx36 and myopia genesis. But this pilot study cannot explain the elaborate pathway of how Cx36 impacting retina function and signal transmission in myopia genesis. Maybe the next step is to investigate how the related neurotransmitter and electric activities change on retina by intervention study. In addition to overexpression or inhabitation of Cx36, Cx36 knockout animal model also can steadily confirm the role of Cx36 in myopia development. Maybe the further investigation on the potential pathway and detailed

infected district in experimental myopia model could provide new prospective and biological targets for myopia therapeutic strategies. However, this study is limited by its failure in capturing the expression changes of Cx36 in the entire process during the experimental period. Detailed changes at more time points can provide us more detailed information and perspective.

Funding

Conflicts of interest
None.

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