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

Guo-Yuan Yang1, Feng-Yang Liu1,2, Xia Li1, Qiu-Rong Zhu1, Bing-Jie Chen1, Long-Qian Liu1

1Department of Ophthalmology, West China Hospital, Sichuan University, Chengdu, Sichuan 610041, China; 2Department 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 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.21 Also, Cx36 is an essential protein in electrical synaptic transmission, which contributes to signal averaging and noise reduction in vision.

Access this article online

Quick Response Code: DOI: 10.1097/CM9.0000000000000319
Website: www.cmj.org

Correspondence to: Prof. Long-Qian Liu, Department of Ophthalmology, West China Hospital, Sichuan University, No. 37 Guoxue Xiang, Chengdu, Sichuan 610041, China
E-Mail: bql565@hotmail.com
Copyright © 2019 The Chinese Medical Association, produced by Wolters Kluwer, Inc. under the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.

Chinese Medical Journal 2019;132(14)
Received: 09-12-2018
Edited by: Ning-Ning Wang
processing. 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 ultrasonography (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 Suwei 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 ScriptcDNA 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 2ΔΔCt 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
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, Alphalnno-tech 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, corneal curvature. At 0-week, eyes in the control group remained hyperopic with only a 1.55 D shift, which indicated that form deprivation induced many myopic changes in SE (Z = –3.38, P < 0.01). Results of the axial

---

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

| Gene   | Primer sequence          | Length (bp) |
|--------|--------------------------|-------------|
| GJD2   | Forward: 5’CAGAGGCGAGATGTTTAGAAG3’  | 186         |
|        | Reverse: 5’GGGACACTGAGCGCTAGAG3’  |             |
| β-actin| Forward: 5’TTCAGGCGACTGTTACTAC3’  | 87          |
|        | Reverse: 5’CAATCTCATCTCGTGTCTG3’  |             |

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 to 0.76)  | 0.10 (0.05 to 0.21)  | –3.37†     | <0.01|
| CC (mm)    | 0.16 (0.06 to 0.23)  | 0 (–0.26 to 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.
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, 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. 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, acts in the electrical coupling between mouse rods and cones, and finishes sophisticated cell-cell communication networks via gap junction channels. 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. 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. First, electrical transmission network via gap junction is an important mode of inter-neuronal communication besides chemical synaptic transmission in the retina. 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. 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. Electoretinogram 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. The activity of Cx36 (as determined by its phosphorylation

---

**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.
level) appears to be regulated by light exposure and dopamine level, two widely accepted elements in eye growth and myopia control. 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. The cyclic adenosine monophosphate level modulates scleral collagen remodeling, which is a critical step in the development of myopia.

This study observed a significant reduction of GJD2 gene mRNA and Cx36 protein expression in a FDM model using guinea pigs. Combining 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 identify 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**

This work was supported by a grant from Department of Science and Technology of Sichuan Province Fund for Medical Sciences (No. 2015SZ0069).

**Conflicts of interest**

None.

**References**

1. Bourne RR, Stevens GA, Smith JL, Flaxman SR, Price H, et al. Causes of vision loss worldwide, 1990–2010: a systematic analysis. Lancet Glob Health 2013;1:e339–e349. doi: 10.1016/S2214-109X(13)70113-X.
2. Holden BA, Fricke TR, Wilson DA, Jong M, Naidoo KS, Sankaridurg P, et al. Vision Loss Expert Group. Global prevalence of myopia and high myopia and temporal trends from 2000 through 2050. Ophthalmology 2016;123:1036–1042. doi: 10.1016/j.ophtha.2016.01.006.
14. Tideman JW, Fan Q, Polling JR, Guo X, Yazar S, Khawaja A, Li YT, Xie MK, Wu J. Association between ocular axial length-related genes and high myopia in a Han Chinese population. Nat Genet 2013;45:314–318. doi: 10.1038/ng.2554.

15. Cheng CY, Schache M, Ikram MK, Young TL, Guggenheim JA, Zhou X, Y, Kumagai K, et al. Large scale international replication and meta-analysis of the 15q14 locus with myopia. J Neurosci 2013;33:1705–1710. doi: 10.1523/JNEUROSCI.5547-03.2004.

16. Kovács-Öller T, Debertin G, Balogh M, Ganczer A, Orbán J, Nyitrai MT. ON pathway mutations increase susceptibility to form-deprivation myopia. Exp Eye Res 2015;137:79–83. doi: 10.1016/j.exer.2015.06.009.

17. Liang Z, Freed MA. Cross inhibition from ON to OFF pathway improves the efficiency of contrast encoding in the mammalian retina. J Neurophysiol 2012;108:2679–2688. doi: 10.1152/jn.00589.2012.

18. Chakraborty R, Park HN, Hanif AM, Siddhu CS, Iuvone PM, Parwise MT. ON pathway mutations increase susceptibility to form-deprivation myopia. Eye Exp Res 2015;137:79–83. doi: 10.1016/j.exer.2015.06.009.

19. Zhang Z, Li H, Liu X, O’Brien J, Ribelayga CP. Cardiac clock control of connexin36 phosphorylation in retinal photoreceptors of the CBA/CaJ mouse strain. Vis Neurosci 2015;32:8009. doi: 10.1017/S0952523815000061.

20. Han Y, Massey SC. Electrical synapses in retinal ON cone bipolar cells: subtype-specific expression of connexins. Proc Natl Acad Sci USA 2005;102:13313–13318. doi: 10.1073/pnas.0505067102.

21. Hartwe et al. Veruki ML. Electrical synapses between all amacrine cells in the retina: function and modulation. Brain Res 2012;1487:160–172. doi: 10.1016/j.brainres.2012.05.060.

22. Aoki C, Kamakura C, Kitamura D, Hara M, et al. Bipolar cell gap junctions serve major signaling pathways in the human retina. Brain Struct Funct 2017;222:2603–2624. doi: 10.1007/s00429-016-1360-4.

23. Biont JJ, Chen X, Macleish PR, O’Brien J, Massey SC. Photoreceptor coupling mediated by connexin36 in the primate retina. J Neurosci 2012;32:4675–4687. doi: 10.1523/JNEUROSCI.1090-11.2012.

24. Pan F, Pan DL, Bloomfield SA, Volgyi B. Connexin36 is required for gap junctional coupling of most ganglion cell subtypes in the mouse retina. J Comp Neurol 2010;518:351–365. doi: 10.1002/cne.21999.

25. Akopian A, Kumar S, Ramakrishnan H, Roy K, Viswanathan S, Bloomfield SA. Targeting neuronal gap junctions in mouse retina offers neuroprotection in glaucoma. J Clin Invest 2017;130:2647–2661. doi: 10.1172/JCI91948.

26. Gong Q, Janowski M, Xie X, Yang G, Liu L. Rasgrf1 mRNA expression in myopic eyes of guinea pigs. Clin Exp Ophthalmol 2016;44:231–237. doi: 10.1111/cxo.12476.

27. Asteriti S, Gargini C, Cangiano L. Connexin 36 expression is required for electrical coupling between mouse rods and cones. Vis Neurosci 2017;34:E006. doi: 10.15760/visneu.2017.000037.

28. Curti S, Hoge G, Nagy JI, Pereda AE. Electrical transmission between mammalian neurons is supported by a small fraction of gap junction channels. J Membr Biol 2012;245:283–290. doi: 10.1007/s00232-012-9449-z.

29. Kovác-Oller T, Debertin G, Balogh M, Ganczer A, Orbán J, Nyitrai M, et al. Connexin36 expression in the mammalian retina: a multiple-species comparison. Front Cell Neurosci 2017;11:65. doi: 10.3389/fncel.2017.00065.

30.能量效果，应避免使用高浓度的溶液或试剂。