Identification of long non-coding RNA and mRNA expression in βB2-crystallin knockout mice

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Abstract. βB2-crystallin (CRYBB2) is expressed at an increased level in the postnatal lens cortex and is associated with cataracts. Improved understanding of the underlying biology of cataracts is likely to be critical for the development of early detection strategies and new therapeutics. The present study aimed to identify long non-coding RNAs (lncRNAs) and mRNAs associated with CRYBB2 knockout (KO)–induced cataracts. RNAs from 3 non-treated mice and 3 CRYBB2 KO mice were analyzed using the Affymetrix GeneChip Mouse Gene 2.0 ST array. A total of 149 lncRNAs and 803 mRNAs were identified to have upregulated expression, including Snora73b, Klk1b22 and Rnu3a, while the expression levels of 180 lncRNAs and 732 mRNAs were downregulated in CRYBB2 KO mice, including Snord82, Snhg9 and Foxn3. This lncRNA and mRNA expression profile of mice with CRYBB2 KO provides a basis for studying the genetic mechanisms of cataract progression.

Introduction

Congenital cataracts area common cause of blindness, with the incidence estimated to be 1-6/10,000 infants in most populations (1,2). There are 13,000-200,000 patients with bilateral congenital cataract who go blind each year worldwide, with an increase of 2,000-40,000 per year (3). The primary clinical manifestation of the disease is the occurrence of lens opacity in the first year (4). Although surgical techniques and visual prognosis have improved, congenital cataracts remain the leading cause of visual disability in children worldwide (5). Previous studies have revealed that almost one-third of congenital cataracts are caused by genetic mutations (6), and 13 genes have previously been confirmed to be associated with congenital cataracts (7). These include crystallin genes [αA-crystallin (CRYAA), αB-crystallin, βA1-crystallin (CRYBA1), βB1-crystallin, βB2-crystallin (CRYBB2), γC-crystallin and γD-crystallin (CRYGD)], membrane transport protein genes [major intrinsic protein (MIP), gap junction protein (GJA3 and GJA8), a cytoskeletal protein gene [beaded filament structural protein 2 (BFSP2)], and transcription factor genes (paired-like homeodomain 3 and heat shock transcription factor 4).

Evidence indicates that gene expression in the lens epithelium is significantly altered during cataract formation. Sheets et al (8) reported the downregulation of CRYAA and CRYBA1/CRYBA3 and the upregulation of the receptor tyrosine kinase adhesion-related kinase (ARK) in the Emory mouse, a well-characterized model of age-dependent cataracts. Furthermore, metallothionein-IIA, osteonectin and ARK are upregulated in cataractous lenses relative to transparent lenses (9-11). Ruotolo et al (12) identified extensive downregulation of genes, including GCS1, GRB7, FST and POLR2E, in the lens associated with the development of age-related cataracts in humans. Although these changes in gene expression are informative, further gene identifications are required to elucidate the molecular mechanism of cataract formation. Crystallins, CRYBB2 in particular, are considered to act primarily as structural proteins of the lens (13). Previously, it was demonstrated that the relative amounts of CRYBB2 protein expression in the lens change markedly, increasing from 12 to 24% (14), suggesting that CRYBB2 serves a contributive function in lens development. Moreover, targeted knockout (KO) of CRYBB2 in mice has been demonstrated to induce age-related (15) and congenital cataracts (16); however, its functional significance is not yet known.

Long non-coding RNAs (lncRNAs) are defined as non-coding RNA molecules >200 nucleotides in length with limited protein coding potential (17,18). Previous studies have indicated that lncRNAs are deregulated in numerous diseases and associated with a wide range of biological processes, such as proliferation, apoptosis and cell migration (19,20). Recently, some lncRNAs have been identified to serve critical functions in eye development and diseases. Shen et al (21) reported that 38 lncRNAs were differentially expressed between transparent and cataractous lenses, among which one of the most abundant lncRNAs, myocardial infarction associated transcript, was specifically upregulated in the plasma fraction of whole blood and the aqueous humor of cataract patients. However, the function of lncRNAs in human lenses remains unknown.
In the present study, differences in lncRNA and mRNA expression between the lenses of untreated mice and CRYBB2 KO-induced cataract mouse models were evaluated. A total of 149 lncRNAs and 803 mRNAs were identified whose expression was upregulated, while the expression levels of a further 180 lncRNAs and 732 mRNAs were downregulated in CRYBB2 KO mouse lenses. These findings suggest a potential function for these lncRNAs and mRNAs in cataract formation.

Materials and methods

Animals. A total of 3 male wild type (WT) and 3 male CRYBB2 KO BALB/c mice (age, 12 weeks old; weight, 25 g) were provided by in Genious Targeting Laboratory, Inc. (Ronkonkoma, NY, USA) (22). Mice with targeted disruption of the CRYBB2 gene were generated at the company by inserting a neo expression cassette to replace the first and second exons, preventing the production of a functional transcript from this locus. Mice were maintained in an animal facility at 25°C, with a relative humidity of 60-70%, under a 12-h light/dark cycle with free access to food and water at the Laboratory Animal Center of the Changhai Hospital, Second Military Medical University (Shanghai, China). All procedures were carried out in accordance with the Chinese legislation on the Use and Care of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (23) and were approved by the Institutional Animal Care and Use Committee of Changhai Hospital, Second Military Medical University (Shanghai, China).

RNA extraction. Following the sacrifice of the mice, the lenses were collected and RNA was isolated from the lenses of mice using the Chomczynski method (24) and was further purified using an RNeasy MinElute Clean-up kit (Qiagen GmbH, Hilden, Germany). The RNA concentration was measured with a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The A260/A280 ratio was 1.8-2.0 and the quality of the RNA was verified by agarose gel electrophoresis.

Microarray processing. LncRNA and mRNA expression profiling was performed using the Affymetrix GeneChip Mouse Gene 2.0 ST array (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocols. Intensities of target hybridization to respective probe features were detected by laser scanning of the array. First, quantile normalization of the microarray data of the 3 untreated and 3 CRYBB2 KO mice was performed. The data was then log2-scale transformed. Hierarchical clustering of the lncRNA and mRNA profiles was performed using Cluster 3.0 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) (25). The normalized expression values of the lncRNAs and mRNAs were centered on the median before unsupervised hierarchical clustering was performed. Clustering was performed with complete linkage and centered Pearson correlation. To estimate the accuracy of the measurements, the coefficient of variance for each measured parameter was determined.
Statistical analysis. Statistical analyses were performed with the use of GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA). The Significance Analysis of Microarray method was used to identify significant gene expression changes between CRYBB2 KO mice and controls (26). P<0.05 was considered to indicate a statistically significant difference.

Results

Analysis of IncRNA expression patterns in CRYBB2 KO mice. The IncRNA expression profiles of lens tissues were compared using unsupervised hierarchical clustering in 3 untreated and 3 CRYBB2 KO mice. As demonstrated in Fig. 1, in total, 329 IncRNAs with a coefficient of variance >0.10 were selected for clustering analysis. Hierarchical clustering of these 329 IncRNAs based on centered Pearson correlation indicated notable differential IncRNA expression in CRYBB2 KO and untreated mice (Fig. 1). Among these IncRNAs, 17 exhibited at least a two-fold change in the CRYBB2 KO mice compared with the untreated mice (all upregulated in CRYBB2 KO mice). A total of 149 out of 329 IncRNAs were upregulated in CRYBB2 KO mice compared with the untreated mice (Table I presents the top 20 most upregulated IncRNAs), whereas 180 out of 329 IncRNAs were downregulated in CRYBB2 KO mice compared with the untreated mice (Table II presents the top 20 most downregulated IncRNAs).

Table I. Top 20 upregulated long non-coding RNAs in βB2-crystallin KO mice.

| Probe set ID | Fold-change (KO/WT) | MGI gene symbol | Gene description | GenBank accession no. |
|--------------|----------------------|-----------------|------------------|----------------------|
| 17296979     | 2.00x10^-7           | Gm10409         | Predicted gene 10409 | NR_033121            |
| 17303368     | 1.00x10^-6           | Gm3002          | α-takusan pseudogene | NR_033388            |
| 17296602     | 2.60x10^-6           | Gm3020          | Predicted gene 3020  | NR_033117            |
| 17430831     | 3.57x10^-3           | Snora73b        | Small nucleolar RNA, H/ACA box 73b | NR_028513 |
| 17303147     | 1.70x10^-6           | Gm3591          | Predicted gene 3591  | Xr_141206            |
| 17434158     | 6.77x10^-3           |                |                  |                     |
| 17337152     | 4.01x10^-3           |                |                  | ENSMUST0000174424   |
| 17424407     | 1.21x10^-2           | 4933409K07Rik   | RIKEN cDNA 4933409K07 gene | NR_033123 |
| 17413061     | 3.08x10^-3           |                |                  |                     |
| 17480922     | 3.86x10^-4           | Mir139          | MicroRNA139NR_029791 |                     |
| 17302054     | 3.04x10^-2           | Snora31         | Small nucleolar RNA, H/ACA box 31 | NR_028481 |
| 17232731     | 3.02x10^-2           | Rnu3a           | U3A small nuclear RNA | NR_002842 |
| 17412952     | 9.63x10^-3           | Gm3893          | Predicted gene 3893  | NR_033506            |
| 17342996     | 2.00x10^-3           | Gm16197         | Predicted gene 16197 | NR_036469            |
| 17421488     | 1.19x10^-2           |                |                  |                     |
| 17348121     | 3.41x10^-2           | 4833419F23Rik   | RIKEN cDNA4833419F23 gene | NR_040328 |
| 17347279     | 3.47x10^-2           |                |                  |                     |
| 17430833     | 1.42x10^-3           | Snora73a        | Small nucleolar RNA, H/ACA box 73a | NR_028512 |
| 17221923     | 2.92x10^-2           |                |                  |                     |
| 17523680     | 4.81x10^-4           | Mir101c         | MicroRNA101cNR_039546 |

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

Analysis of mRNA expression patterns in CRYBB2 KO mice. The mRNA expression profiles of lens tissues were compared using unsupervised hierarchical clustering in the 3 untreated and 3 CRYBB2 KO mice. In total, 1,535 mRNAs with a coefficient of variance >0.10 were selected for clustering analysis. Hierarchical clustering of these 1,535 mRNAs based on centered Pearson correlation indicated notable differential mRNA expression between CRYBB2 KO and untreated mice (Fig. 2). Among these mRNAs, 52 exhibited at least a two-fold change in the CRYBB2 KO mice compared with the untreated mice (all upregulated in CRYBB2 KO mice). A total of 803 out of 1,535 mRNAs were upregulated in CRYBB2 KO mice compared with the untreated mice (Table III presents the top 20 most upregulated mRNAs), whereas 732 out of 1,535 mRNAs were downregulated (Table IV presents the top 20 most downregulated mRNAs).

Discussion

In the present study, the IncRNA and mRNA profiles of untreated and CRYBB2 KO cataractous lenses were evaluated. A total of 149 IncRNAs and 803 mRNAs were identified to be upregulated, while 180 IncRNAs and 732 mRNAs were identified to be downregulated in CRYBB2 KO mice lenses, implying a potential role of these IncRNAs and mRNAs in cataract formation.

In previous research, an increasing number of IncRNAs have been identified and associations between IncRNAs and...
Table II. Top 20 downregulated long non-coding RNAs in βB2-crystallin KO mice.

| Probe set ID | P-value | Fold-change (KO/WT) | MGI gene symbol | Gene description | GenBank accession no. |
|--------------|---------|---------------------|------------------|------------------|----------------------|
| 17251898     | 4.62x10^{-2} | 0.86 | Mir324 | MicroRNA 324 | NR_029758 |
| 17547715     | 4.84x10^{-2} | 0.86 | - | - | ENSMUST00000117972 |
| 17468138     | 4.35x10^{-2} | 0.85 | - | - | ENSMUST00000145420 |
| 17329209     | 4.58x10^{-2} | 0.85 | A830060N17 | Uncharacterized LOC328646 | NR_046162 |
| 17365718     | 3.37x10^{-2} | 0.84 | - | - | ENSMUST00000162724 |
| 17403967     | 3.43x10^{-2} | 0.84 | - | - | ENSMUST00000158662 |
| 17362668     | 3.62x10^{-2} | 0.84 | - | - | ENSMUST00000169060 |
| 17315735     | 3.71x10^{-2} | 0.84 | - | - | ENSMUST00000160968 |
| 17527984     | 4.10x10^{-2} | 0.84 | A730043L09 | Uncharacterized protein A730043L09 | NR_040769 |
| 17278612     | 4.40x10^{-2} | 0.84 | Mir342 | MicroRNA342 | NR_029771 |
| 17448958     | 2.17x10^{-2} | 0.83 | - | - | ENSMUST00000158856 |
| 17269866     | 3.17x10^{-2} | 0.83 | - | - | ENSMUST00000102272 |
| 17225173     | 3.59x10^{-2} | 0.83 | Snord82 | Small nucleolar RNA, C/D box 82 | NR_002851 |
| 17303480     | 3.98x10^{-2} | 0.83 | - | - | ENSMUST00000128545 |
| 17532275     | 4.53x10^{-2} | 0.83 | - | - | ENSMUST00000082463 |
| 17345664     | 4.84x10^{-2} | 0.83 | - | - | ENSMUST00000122623 |
| 17280661     | 2.01x10^{-2} | 0.82 | F730043M19Rik | RIKEN cDNA F730043M19 gene | NR_015602 |
| 17395003     | 2.17x10^{-2} | 0.82 | - | - | ENSMUST00000133525 |
| 17342024     | 2.90x10^{-2} | 0.82 | Snhg9 | Small nucleolar RNA host gene (non-protein coding) 9 | NR_027900 |
| 17232800     | 3.09x10^{-2} | 0.82 | - | - | ENSMUST00000104610 |

KO, knockout; WT, wild type; MGI, mouse genome informatics database.

Table III. Top 20 upregulated mRNAs in βB2-crystallin KO mice.

| Probe set ID | P-value | Fold-change (KO/WT) | MGI gene symbol | Gene description | GenBank accession no. |
|--------------|---------|---------------------|------------------|------------------|----------------------|
| 17477347     | 3.60x10^{-2} | 7.22 | Klk1b22 | Kallikrein 1-related peptidase b22 | NM_010114 |
| 17303018     | 1.00x10^{-7} | 7.14 | Gm3500 | Predicted gene 3500 | NM_001256886 |
| 17296943     | 2.52x10^{-4} | 5.78 | - | - | ENSMUST00000163719 |
| 17303117     | 1.00x10^{-7} | 5.58 | Gm3696 | Predicted gene 3696 | ENSMUST00000167923 |
| 17296896     | 4.00x10^{-7} | 5.14 | Gm5796 | Predicted gene 5796 | NM_001029930 |
| 17548311     | 1.22x10^{-5} | 4.56 | Gm3579 | Predicted gene 3579 | AY140896 |
| 17373996     | 1.35x10^{-4} | 4.53 | BC048594 | cDNA sequence BC048594 | BC048594 |
| 17331088     | 4.22x10^{-5} | 4.48 | Gm19797 | Predicted gene 19797 | XM_003085996 |
| 17303024     | 3.66x10^{-4} | 4.13 | Gm10021 | Predicted gene 10021 | AK084071 |
| 17413352     | 5.14x10^{-3} | 3.46 | Car9 | Carbonic anhydrase 9 | ENSMUST0000030183 |
| 17296849     | 9.00x10^{-6} | 3.4 | Gm2897 | Predicted gene 2897 | NM_001177715 |
| 17335467     | 1.21x10^{-2} | 3.22 | Cdkn1a | Cyclin-dependent kinase inhibitor 1A (P21) | NM_007669 |
| 17412962     | 7.94x10^{-3} | 3.2 | Gm3893 | Predicted gene 3893 | BC059060 |
| 17496857     | 2.59x10^{-2} | 3.16 | Cox6a2 | Cytochrome c oxidase, subunit VI a, polypeptide 2 | NM_009943 |
| 17466743     | 9.47x10^{-4} | 3.13 | Npvl | Neuropeptide VF precursor | ENSMUST00000031853 |
| 17331078     | 3.77x10^{-4} | 3.06 | Tmem45a | Transmembrane protein 45a | NM_019631 |
| 17296836     | 2.00x10^{-6} | 3.01 | Gm5458 | Predicted gene 5458 | NM_001024706 |
| 17280292     | 5.05x10^{-4} | 2.93 | - | - | ENSMUST00000169148 |
| 17296595     | 2.52x10^{-3} | 2.9 | D830030K20Rik | RIKEN cDNA D830030K20 gene | ENSMUST00000169218 |
| 17303315     | 5.57x10^{-3} | 2.78 | Gm5797 | Predicted gene 5797 | ENSMUST00000100886 |

KO, knockout; WT, wild type; MGI, mouse genome informatics database.
numerous diseases, including cardiovascular and neurodegeneration diseases, have been reported (27). The roles of IncRNAs in cancer development are being studied (28,29). However, the function of IncRNAs in disease, particularly in cataracts, has not yet been reported. To the best of our knowledge, the current study presents the first report on differential IncRNA expression in a cohort of mice with or without CRYBB2 KO. Through an analysis of lenses, it was identified that 329 IncRNAs were differentially expressed in CRYBB2 KO and untreated mice, suggesting that IncRNAs may serve critical functions in cataract formation. Among the top 20 most upregulated IncRNAs, five were predicted genes and a further six were unnamed. Among the top 20 most downregulated IncRNAs, 13 IncRNAs were unnamed and the others were known (identified with Mouse Genome Informatics gene symbols). These results indicated that these IncRNAs were linked with CRYBB2-associated cataract formation. Notably, the expression changes of IncRNAs in the upregulated group (maximum change, 5.84-fold) were higher compared with those in the downregulated group (maximum change, 0.86-fold), suggesting a higher susceptibility of IncRNAs to be upregulated rather than downregulated in cataracts.

Differential mRNA expression was also examined in the cohort of mice with or without CRYBB2 KO. Through an analysis of lenses, it was identified that 1,535 mRNAs were differentially expressed between CRYBB2 KO and untreated mice. Among the top 20 most upregulated mRNAs, 10 mRNAs were predicted genes and two mRNAs were unnamed. These results indicated that these mRNAs may serve critical functions in cataract formation. Notably, the expression changes of mRNAs in the upregulated group (maximum change, 7.22-fold) were higher compared with those in the downregulated group (maximum change, 0.86-fold), suggesting a higher susceptibility of mRNAs to be upregulated rather than downregulated in cataracts.

A previous limited microarray survey with a panel of cell cycle-regulated genes illustrated that irradiation with protons altered the gene expression pattern of human lens epithelial cells (30), such as cyclin-dependent kinase inhibitor 1 (CDKN1A), which codes for a protein that is involved in several pathways functionally associated with linear energy transfer-responsive radiation damage. Cytochrome C oxidase 6A2 (COX6A2) was identified to be upregulated during cataract development in mice with a mutation in MIP, a functional water channel that serves a key role in establishing lens fiber cell architecture and is associated with inherited and age-related forms of cataracts (31). Consistent with these results, the present study also identified upregulated expression of CDKN1A and COX6A2 in a CRYBB2 KO-induced cataract mice model. Furthermore, Fas-mediated apoptosis in human lens epithelial cells of cataracts is associated with diabetic retinopathy (32), suggesting a role for Fas in cataract formation, which is contrary to the finding of the present study that Fas was downregulated in a CRYBB2 KO-induced cataracts mouse model (data not shown). BFSP2, a gene for a lens-specific beaded filament structural protein, was down-regulated in CRYBB2 KO-induced cataract mice (33,34), which is in agreement with the findings of the present study: BFSP2 expression is restricted to the lens fiber cells, and a deletion mutation of BFSP2 is associated with
cataracts. CRYGD mutation has previously been observed to cause autosomal dominant congenital cerulean cataracts, suggesting an inhibitory role of CRYGD in cataract formation (35). This is consistent with the current findings that CRYGD is downregulated in a CRYBB2 KO-induced cataracts mouse model (data not shown).

The present study has some limitations, including the relatively small number of mice in each cohort, and the fact that only RNA samples from the lens were utilized for hybridizations. Furthermore, the differentially expressed lncRNAs and mRNAs require further clarification in future investigations.

In conclusion, knowledge of the changes in lncRNA and mRNA expression associated with cataracts may contribute to a better understanding of the opacification process. The findings of the present study demonstrate that there are notable lncRNA and mRNA differences between mice with or without CRYBB2 KO induction. The data indicate that the response of the lens to the development of CRYBB2 KO-related cataract is characterized by an extensive upregulation of numerous mRNAs and lncRNAs.

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**Availability of data and materials**
All data generated or analyzed during this study are included in this published article.

**Authors' contributions**
YJ conceived of and designed the experiments. KX, H-XR and W-JL performed the experiments and analyzed the data. W-JL and KX obtained the reagents, materials and analysis tools. YJ and W-JL wrote the study. All authors read and approved the final study.

**Table IV. Top 20 downregulated mRNAs in βB2-crystallin KO mice.**

| Probe set ID | P-value | Fold-change (KO/WT) | MGI gene symbol | Gene description | GenBank accession no. |
|-------------|---------|---------------------|-----------------|------------------|----------------------|
| 17256388    | 3.86x10^{-2} | 0.86 | Ttc25 | Tetramericopeptide repeat domain 25 | NM_028918 |
| 17283203    | 4.91x10^{-2} | 0.86 | Foxn3 | Forkhead box N3 | ENSMUST0000046859 |
| 17357502    | 4.94x10^{-2} | 0.86 | Cpsf7 | Cleavage and polyadenylation specific factor 7 | NM_172302 |
| 17440775    | 4.31x10^{-2} | 0.86 | Dao | D-amino acid oxidase | ENSMUST00000112292 |
| 17473796    | 4.34x10^{-2} | 0.86 | Rps5 | Ribosomal protein S5 | NM_009095 |
| 17528663    | 4.79x10^{-2} | 0.86 | Polr2m | Polymerase (RNA) II (DNA directed) polypeptide M | NM_178602 |
| 17231003    | 4.52x10^{-2} | 0.85 | Mfsd7b | Major facilitator superfamily domain containing 7B | NM_001081259 |
| 17219005    | 4.68x10^{-2} | 0.85 | Creg1 | Cellular repressor of E1A-stimulated genes 1 | NM_011804 |
| 17225580    | 4.98x10^{-2} | 0.85 | Olfr1415 | Olfactory receptor 1415 | NM_001011525 |
| 17256716    | 4.83x10^{-2} | 0.85 | Rundc1 | RUN domain containing 1 | NM_172566 |
| 17291143    | 3.31x10^{-2} | 0.85 | - | - | AK029074 |
| 17304186    | 4.98x10^{-2} | 0.85 | Plac9 | Placenta specific 9 | NM_207229 |
| 17358640    | 4.57x10^{-2} | 0.85 | Mbl2 | Mannose-binding lectin (protein C) 2 | ENSMUST0000025797 |
| 17368499    | 4.89x10^{-2} | 0.85 | Dbh | Dopamine beta hydroxylase | ENSMUST0000009910 |
| 17425160    | 3.58x10^{-2} | 0.85 | Erp44 | Endoplasmic reticulum protein 44 | NM_029572 |
| 17451345    | 4.98x10^{-2} | 0.85 | 2900026A02Rik | RIKEN cDNA 2900026A02 gene | NM_172884 |
| 17501800    | 3.53x10^{-2} | 0.85 | Hapln4 | Hyaluronan and proteoglycan link protein 4 | NM_177900 |
| 17499224    | 3.82x10^{-2} | 0.85 | F10 | Coagulation factor X | NM_001242368 |
| 17504309    | 3.96x10^{-2} | 0.85 | Cced113 | Coiled-coil domain containing 113 | NM_172914 |
| 17502071    | 4.01x10^{-2} | 0.85 | - | - | ENSMUST0000050921 |

KO, knockout; WT, wild type; MGI, mouse genome informatics database.
Ethics approval and consent to participate

All procedures were carried out in accordance with the Chinese legislation on the Use and Care of Laboratory Animals and the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research (23) and were approved by the Institutional Animal Care and Use Committee of Shanghai Hospital, Second Military Medical University (Shanghai, China).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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