

Simultaneous expression of two pathogenic genes in four Chinese patients affected with inherited retinal dystrophy

Xiao-Zhen Liu¹, Tian-Chang Tao², Hong Qi¹, Shan-Na Feng¹, Ning-Ning Chen¹, Lin Zhao¹, Zhi-Zhong Ma¹, Gen-Lin Li², Li-Ping Yang¹

¹Department of Ophthalmology, Peking University Third Hospital, Beijing Key Laboratory of Restoration of Damaged Ocular Nerve, Beijing 100191, China

²Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing Ophthalmology & Visual Sciences Key Lab, Beijing 100730, China

Correspondence to: Li-Ping Yang. Department of Ophthalmology, Peking University Third Hospital, Beijing Key Laboratory of Restoration of Damaged Ocular Nerve, Beijing 100191, China. alexlipingyang@bjmu.edu.cn; Gen-Lin Li. Beijing Tongren Eye Center, Beijing Tongren Hospital, Capital Medical University, Beijing 100730, China. ligenglin@263.net
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Abstract

• **AIM:** To describe the complex, overlapping phenotype of four Chinese patients with inherited retinal dystrophies (IRDs) who harbored two pathogenic genes simultaneously.

• **METHODS:** This retrospective study included 4 patients affected with IRDs. Medical and ophthalmic histories were obtained, and clinical examinations were performed. A specific Hereditary Eye Disease Enrichment Panel (HEDEP) based on exome capture technology was used for genetic screening.

• **RESULTS:** Four patients were identified to harbor disease-causing variants in two different genes. Patient retinitis pigmentosa (RP) 01-II:1 exhibited both classical *ABCA4*-induced Stargardt disease (STGD) 1 and *USH2A*-associated RP, patient RP02-III:2 exhibited both classical *ABCA4*-induced STGD1 and *CDH23*-associated RP, patient RP03-II:1 exhibited both *USH2A*-induced autosomal recessive retinitis pigmentosa (arRP) syndrome and *SNRNP200*-induced autosomal dominant retinitis pigmentosa (adRP), and patient RP04- II:2 exhibited *USH2A*-induced arRP syndrome and *EYS*-induced arRP at the same time.

• **CONCLUSION:** Our study demonstrates that genotype-phenotype correlations and comprehensive genetic screening is crucial for diagnosing IRDs and helping family planning for patients suffering from the disease.

• **KEYWORDS:** inherited retinal dystrophies; Hereditary Eye Disease Enrichment Panel; retinitis pigmentosa; Stargardt disease; two pathogenic genes

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INTRODUCTION

Inherited retinal dystrophies (IRDs) are a class of monogenic, vision-impairing diseases resulting from photoreceptor and retinal pigment epithelium (RPE) degeneration. The spectrum of IRDs consists of diseases that predominantly affect the central retina [such as Stargardt disease (STGD)], the peripheral retina [such as retinitis pigmentosa (RP)], or both [such as Leber congenital amaurosis (LCA)]^[1-2]. A number of patients do not display specific clinical phenotypes associated with a particular IRD, but exhibit overlapping phenotypes that are consistent with more than one particular disease, and intra-familial variability and incomplete penetrance are not uncommon, leaving a definitive and precise clinical diagnosis challenging^[2]. The predictive power of either targeted genetic screening or clinical examination alone, however, remains limited. Fortunately, increasing accessibility of gene sequencing has provided both scientists and clinicians with the ability to make valuable genotype-phenotype correlations^[1-3]. STGD1, is the most common type of all STGD cases, resulting from variants in the adenosine triphosphate (ATP) binding cassette subfamily A member *ABCA4* gene^[4]. With a prevalence of 1:8000 to 1:10 000^[5-6], STGD1 is also the most frequent inherited macular disorder. Typical representations of STGD1 are juvenile to young-adult onset and progressive central vision impairment and a varying extent of atrophy of the RPE around the macular and perimacular region. The fundus of STGD1 patients may show the appearance of “beaten metal” or “snail slime,” with representative whitish-yellow flecks restricted to the fovea or parafoveal macula in the relatively early stage, and widespread RPE and chorioretinal atrophy in the late period^[5].

RP is the most frequency subtype of IRDs with typical clinical manifestation of night blindness followed by decreasing visual fields, leading to tunnel vision and ultimately blindness^[7]. The clinical hallmarks of RP include bone spicule deposits; attenuated retinal blood vessels; optic disc pallor; visual field loss; and abnormal, diminished, or nonrecordable electroretinographic (ERG) responses^[8]. The incidence rate of RP in America and Europe is about 1:3500 to 1:4000, respectively^[9], and is 1:1000 in northern China^[10]. Over 80 genes have been reported to be associated with non-syndromic RP (RetNet; <http://www.sph.uth.tmc.edu/Retnet/>). In approximately one third of RP patients, retinal degeneration appears as part of other systemic diseases, such as Usher syndrome *etc.*

Here, we used a specific Hereditary Eye Disease Enrichment Panel (HEDEP) based on exome capture technology to screen probands with IRDs and detected that four patients carried two different IRD-causing genes. Previously, the complex phenotypes caused by the simultaneous expression of STGD1 and other IRDs such as congenital stationary night blindness, retinoblastoma, or X-linked ocular albinism have been described^[3,11-12]. This study describes the clinical manifestations of two patients who simultaneously demonstrated STGD1 phenotype due to *ABCA4* variants and the characteristic RP phenotype due to *USH2A* or *CDH23* variants respectively. We also report two cases diagnosed with Usher syndrome, aside from *USH2A* gene the two patients also affected with *SNRNP200* induced autosomal dominant retinitis pigmentosa (adRP) or *EYS* induced autosomal recessive retinitis pigmentosa (arRP) respectively.

SUBJECTS AND METHODS

Ethical Approval The study conforms to the tenets of the Declaration of Helsinki. The Peking University Third Hospital Medical Ethics Committee approved all experiments including patient DNA and that of their relatives (No.2012093). Informed consent form was signed by all participants or guardians on behalf of the minors/child participants, and the Ethics Committees approved this consent procedure.

Patients Four Chinese Han patients suffering from IRD from the Department of Ophthalmology, Peking University Third Hospital, and Beijing Tongren Eye Center were recruited. A detailed family history was obtained from the probands and/or their relatives. The standard ophthalmic examinations such as best corrected visual acuity (BCVA) with E decimal charts, slit-lamp biomicroscopy, dilated indirect ophthalmoscopy, fundus photography, fundus autofluorescence (FAF) or fluorescent angiography (FFA), optical coherence tomography (OCT), and ERG were performed on the affected individuals, if possible. The ERG protocol complied with the standards published by the International Society for Clinical Electrophysiology of Vision (ISCEV).

The diagnosis of RP was based on the criteria listed above and previously^[13]. Patients were diagnosed with STGD1 based on the criteria described by Fishman^[14]. The patients with STGD1 were further classified as having one of the four stages of the disease^[14]. In stage I, patients showed an atrophic-appearing “beaten-bronze” foveal appearance and/or parafoveal whitish-yellow flecks. In stage II, patients exhibited numerous whitish-yellow flecks throughout the posterior pole. In stage III, patients had resorption of the flecks and extensive atrophy of the choriocapillaris in the macula. In stage IV, patients presented with extensive chorioretinal atrophy over the entire fundus^[5].

Mutation Screening Blood samples were obtained from the patients and/or relatives. The genomic DNA was extracted using standard protocols (Omega Bio-Tek Inc, Norcross, GA, USA). The HEDEP was used to target the protein coding regions of 441 hereditary eye disease genes, covering 290 IRDs associated genes. Fifty micrograms of genomic DNA from the patient was used for targeted exome capture and high-throughput sequencing. Targeted gene enrichment, high-throughput sequencing, and data analysis were performed as described previously^[13].

Mutation Validation The identified variants were confirmed by Sanger sequencing on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sanger sequencing was also employed to determine whether the variant co-segregated with the disease phenotype in available family members and in healthy controls. Primers were designed using the primer website (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

RESULTS

In total, four Chinese families with IRDs were recruited in this study. They were all diagnosed with RP, and two of them exhibited overlapping manifestations of STGD1. The detailed clinical information of these four probands is summarized in Table 1.

The probands were subjected for targeted exome capture and high throughput sequencing. We generated 1.5 Gb of sequences with more than 300× coverage. The generated sequence covered an average of 99.8% of the targeted bases, which is sufficient to pass the thresholds for calling single nucleotide polymorphisms (SNPs) and short insertions or deletions (indels). We filtered all the variants with the ethnic Han Chinese Beijing available in the 1000 Genomes Project (<http://www.1000genome.org>) and the Han Chinese Beijing SNPs in the dbSNP131 and found the potential pathogenic variants. Sanger sequencing validation was performed in available family members. The probands were detected to harbor two different disease-causing RP-or/and STGD1-associated genes (Table 2).

Family RP01 The proband RP01-II:1, a 23-year-old man, presented to the clinicians with macular dystrophy, his BCVA

Table 1 Detailed clinical information of four probands

Patient ID	Gender	Clinical diagnosis	Age (y) at exam; onset	History	BCVA (OD; OS)	Fundus features
RP01-II:1	Male	RP + STGD1	23; childhood	Visual impairment and night blindness since childhood; negative family history	0.05; 0.05	Bilateral retinal arteriolar attenuation, widespread RPE atrophy, pigment deposition, bilateral beaten-bronze appearing macular lesions with yellow flecks around macula, pale optic disc
RP02-III:2	Male	arRP + STGD1	25; 10	Decreased visual acuity for about 14y; mild night blindness since childhood; his elder sister was also affected	0.05; 0.05	Bilateral retinal arteriolar attenuation, widespread RPE atrophy; bilateral beaten-bronze appearing macular lesions with yellow flecks around macula, pale optic disc
RP03-II:1	Female	RP syndrome	48; 7	Night blindness for over 40y; hearing impairment gradually; negative family history	0.6; 0.15	Bilateral retinal arteriolar attenuation, widespread RPE atrophy, pigment deposition, grey flecks around macular area, pale optic disc
RP04-II:2	Female	RP syndrome	47; 10	Night blindness for over 40y; decreased visual acuity for about 10y; hearing impairment gradually; negative family history	0.1; 0.2	Bilateral retinal arteriolar attenuation, widespread RPE atrophy; pale optic disc, cataract in both eyes

RP: Retinitis pigmentosa; STGD1: Stargardt disease; BCVA: Best corrected visual acuity; OD: Right eye; OS: Left eye.

were found to be 0.05 bilaterally with no family history. He complained of visual impairment and night blindness since childhood. Fundus examination showed beaten-bronze appearing macular lesions with yellow flecks around the macula (Figure 1A). An FAF image showed a central dark hypofluorescent area corresponding to RPE atrophy (Figure 1B). FFA presented with a typical dark choroid, hyperfluorescence in the poster pole and periphery retina, and massive vascular leakage (Figure 1C, 1D); OCT demonstrated thinning of the retina both at the poster pole and periphery (Figure 1E). Multifocal ERG revealed that both the rod and cone responses were markedly reduced (Figure 1F, 1G). Full-field ERG showed undetectable rod responses consistent with the symptom of night blindness (Figure 1H). Based on the above evidence, the patient was initially diagnosed with STGD1 at stage III. Aside from typical STGD1 features, the patient also demonstrated retinal widespread RPE atrophy, and bone-spicule pigmentation in the midperiphery retina, arteriolar attenuation and the undetectable rod responses, corresponding to characteristic RP.

Genetic studies showed the patient harbored compound heterozygotes for both *ABCA4* (c.6088C>T, p.R2030* and c.5318C>T, p.A1773V) and *USH2A* (c.4758+3A>G and c.1624A>G, p.S542G) genes; the variants p.R2030* and p.S542G were inherited from his father, while the variants p.A1773V and c.4758+3A>G were inherited from his mother (Figure 1I). These variants were absent in 100 matched normal controls. Both variants p.R2030* and p.A1773V of *ABCA4* have been reported as pathogenic^[15-17] and are pathogenic hotspots in STGD1 patients. The splicing variant c.4758+3A>G in *USH2A* was previously reported in patients affected with Usher syndrome^[18-19] and was a pathogenic hotspot in Chinese RP patients in our study. The variant p.S542G was novel, it affected a conserved serine (Figure 1J), and bioinformatics analysis predicted it to be disease-causing. The

variant p. S542G localized to the 1st laminin EGF-like modules (LE domains) of usherin^[20-21]. The LE domain consists of repeat units of 60 amino acids including 8 conserved cysteines^[22]. Usherin integrated into the structural architecture of basement membranes *via* interaction of the LE domain of usherin with the 7S domain of type IV collagen, and amino acid substitutions within the b loop of the first and fourth LE modules disrupt the usherin/type IV collagen interaction^[23]. Considering the clinical manifestation and genetic identification, the patient was affected with an overlapping manifestation of both STGD1 and RP.

Family RP02 In family RP02, the proband RP02-III:2, a 25-year-old man, presented to the clinician with macular dystrophy and irretrievable vision loss. His BCVA was found to be 0.05 bilaterally. His elder sister was also affected. The patients complained of visual defects since childhood, while they also complained of nyctalopia (Table 1). Fundus examination showed retinal arteriolar attenuation, widespread RPE atrophy, bilateral beaten-bronze appearing macular lesions with yellow flecks around macula, and tiny gray dots throughout the retina (Figure 2A, 2B). His FFA presented with hyperfluorescence in the poster pole and mid-peripheral retina due to RPE atrophy; the hypofluorescent dots corresponded to the pigment deposition (Figure 2C, 2D). OCT showed thinning of the outer retina (Figure 2E). The latest full-field ERG of the patient showed markedly reduced rod and cone responses (Figure 2F). No hearing problem was identified (Figure 2G, 2H). From the clinical manifestations, this patient also exhibited overlapping phenotypic traits of RP and STGD1 simultaneously.

Genetic study demonstrated that the patients both carried two compound heterozygous variants (c.157G>T, p.E53* and c.5881G>A, p.G1961R) in the *ABCA4* gene and compound heterozygotes (c.7145G>A, p.R2382Q and c.9617G>A, p.R3206H) in the *CDH23* gene, respectively (Figure 2I). The

Table 2 Variants identified in the four probands

Patient ID	Gene	Genotype	Allele exon nucleotide protein	Computational prediction			MAF in ExAC	MAF in gnomAD	Reference
				SIFT	Polyphen-2	Mutation Taster			
RP01-II:1	<i>ABCA4</i>	Compound heterozygous	exon 38 c.5318C>T	-	-	-	0.0001153	NA	Stenirri et al, 2008
			exon 44 c.6088C>T	-	-	-	0.00002471	NA	Lewis et al, 1999
RP02-III:2	<i>USH2A</i>	Compound heterozygous	exon 9 c.1624A>G	Not tolerated	Possibly damaging	Disease-causing	NA	NA	Novel
			IVS22 c.4758+3A>G	-	-	-	0.001523	0.001453	Aparisi et al, 2014
RP02-III:2	<i>ABCA4</i>	Compound heterozygous	exon 2 c.157G>T	-	-	-	NA	NA	Jiang et al, 2016
			exon 42 c.5881G>A	-	-	-	0.00008244	0.0000969	Riveiro-Alvarez et al., 2006
RP03-II:1	<i>CDH23</i>	Compound heterozygous	exon 49 c.7145G>A	-	-	-	NA	NA	Miyagawa et al, 2013
			exon 66 c.9617G>A	Not tolerated	Probably damaging	Disease-causing	NA	NA	Novel
RP03-II:1	<i>USH2A</i>	Compound heterozygous	IVS22 c.4758+3A>G	-	-	-	0.001523	0.001453	Aparisi et al, 2014
			exon 37 c.7068T>G	-	-	-	0.0007831	0.0005166	Miyagawa et al, 2013
RP04-II:2	<i>SNRNP200</i>	Heterozygous	exon 16 c.2042G>A	-	-	-	NA	NA	Benaglio et al, 2011
			IVS22 c.4758+3A>G	-	-	-	0.001523	0.001453	Aparisi et al, 2014
RP04-II:2	<i>USH2A</i>	Compound heterozygous	exon 37 c.6986C>A	-	-	-	0.00002474	NA	Novel
			exon 16 c.2530C>T	-	-	-	NA	NA	Novel
RP04-II:2	<i>EYS</i>	Homozygous	exon 16 c.2530C>T	-	-	-	NA	NA	Novel
			exon 16 c.2530C>T	-	-	-	NA	NA	Novel

ExAC: Exome aggregation consortium; GnomAD: The genome aggregation database; MAF: Minor allele frequency.

variant p.G1961R in *ABCA4* and p.R2382Q in *CDH23* were inherited from their mother while the remaining variants from their father. All two variants p.E53* and p.G1961R of *ABCA4* were previously reported to cause STGD1 and cone-rod dystrophy^[5,24-25]. The variant p.R2382Q of *CDH23* was previously detected in patients affected with deafness^[26]. The missense substitution c.9617G>A (p.R3206H) was novel, but the variant p.R3206C was reported to be disease-causing previously^[27]. The variant p.R3206H affected a conserved arginine (Figure 2J) and was predicted to be detrimental. The *CDH23* gene encoding calcium-dependent cell-cell adhesion glycoproteins^[28]. There is no doubt that the two variants in *ABCA4* led to macular disease, and it is probable that the compound heterozygous variants in *CDH23* contributed to the RP manifestations.

Family RP03 The initial symptom of subject RP03-II:1 was night blindness since childhood, followed by progressive constriction of peripheral visual field. Her 12-year-old daughter was also affected with night blindness. The proband's BCVA was found to be 0.6 in the right and 0.15 in the left eye. This difference was due to the macular epiretinal membrane in her left eye. Fundus examination showed the classic symptoms of RP, consisting of retinal arteriolar attenuation, waxy pallor of the optic discs, and scattered bone-spicule pigmentation around the mid-peripheral retina (Figure 3A, 3B). Her FFA showed scattered hyperfluorescent RPE in the poster pole and mid-peripheral retina (Figure 3C, 3D), and OCT showed loss of inner-segment ellipsoid and outer nuclear layer in the retina (Figure 3E, 3F). Full-field ERG showed markedly reduced rod responses (Figure 3G). Pure tone audiometry examination showed that the patient has serious hearing impairment (Figure 3H, 3I).

Genetic study showed that the proband carried the compound heterozygotes including c.7068T>G (p.N2356K) and c.4758+3A>G in *USH2A* and a heterozygous variant c.2042G>A (p.R681H) in *SNRNP200* (Figure 3J). Both c.4758+3A>G and p.N2356K were pathogenic hotspots in Chinese RP and Usher syndrome patients and have previously been reported to cause deafness^[18-19,26,29]. The residue Arg681 is phylogenetically very well conserved, and its replacement was detected to cause adRP previously^[30]. The proband's daughter inherited the variant p.R681H from her mother and had nyctalopia since childhood, her fundus examination showed mild RP symptoms (Figure 3K) and FFA shows scattered hyperfluorescent RPE in the poster pole (Figure 3L), indicating the pathogenicity of the p.R681H in this family. Our results showed that two pathogenic genes, *SNRNP200* and *USH2A*, mutated simultaneously in the proband affecting with RP syndrome.

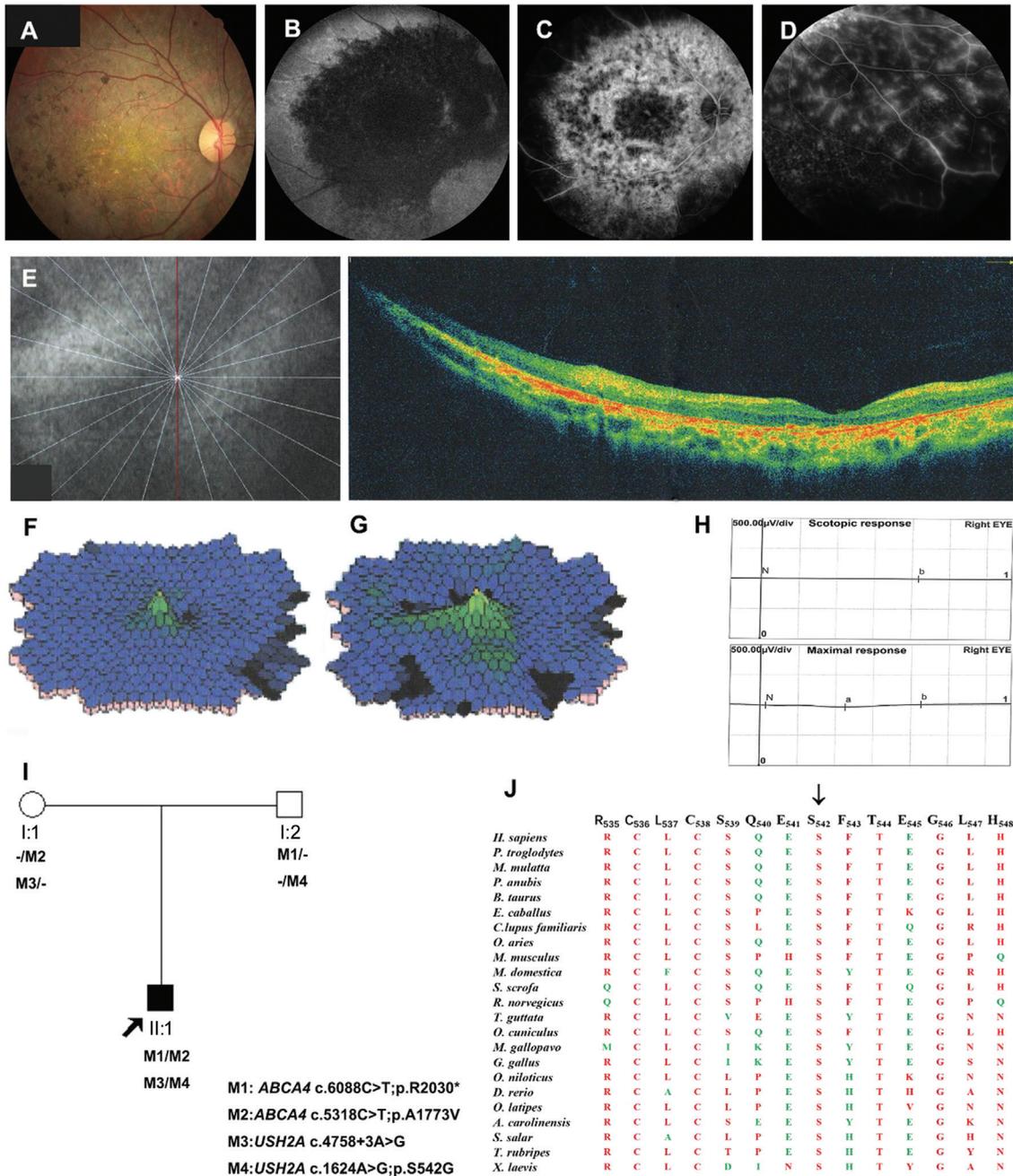


Figure 1 Clinical characteristics of the patient RP01-II:1 A: Fundus examination of the patient’s right eye shows beaten-bronze appearing macular lesions with yellow flecks around the macula, retinal arteriolar attenuation, widespread RPE atrophy, and bone-spicule pigmentation in the midperiphery retina; B: A FAF image of the right eye shows a central dark hypofluorescent area corresponding to chorioretinal and RPE atrophy; C, D: FFA presents with a typical dark choroid, hyperfluorescence in the poster pole due to RPE atrophy, hypofluorescent dots corresponding to the yellow flecks observed in the fundus examination, and massive leakage in the periphery retina; E: OCT demonstrates thinning of the retina; F, G: Multifocal ERG showed that both the cone and rod responses were markedly reduced; H: Full-field ERG shows that both cone and rod responses were markedly reduced; I: Pedigree of family RP01. The filled symbols represent affected individuals; unfilled symbols is unaffected individuals. Squares signify men; circles is women. An arrow marks the index patient; - means a normal allele; J: Multiple orthologous sequence alignment of *USH2A* around the S542 amino acid in 23 species.

Family RP04 Apart from nyctalopia for approximately 40y, subject RP04-II:2 complained of decreased visual acuity and hearing loss in the recent 10y. Her BCVA was 0.1 in the right side and 0.2 in the left side, which was also contributed by cataract. Fundus examination displayed the typical symptoms

of RP, such as bilateral widespread RPE atrophy and retinal arteriolar attenuation, which was not distinguished clearly because of the cataract lesion (Figure 4A, 4B). Her FFA demonstrated scattered hyperfluorescent RPE (Figure 4C, 4D), and the OCT showed thinning of the outer retina (Figure

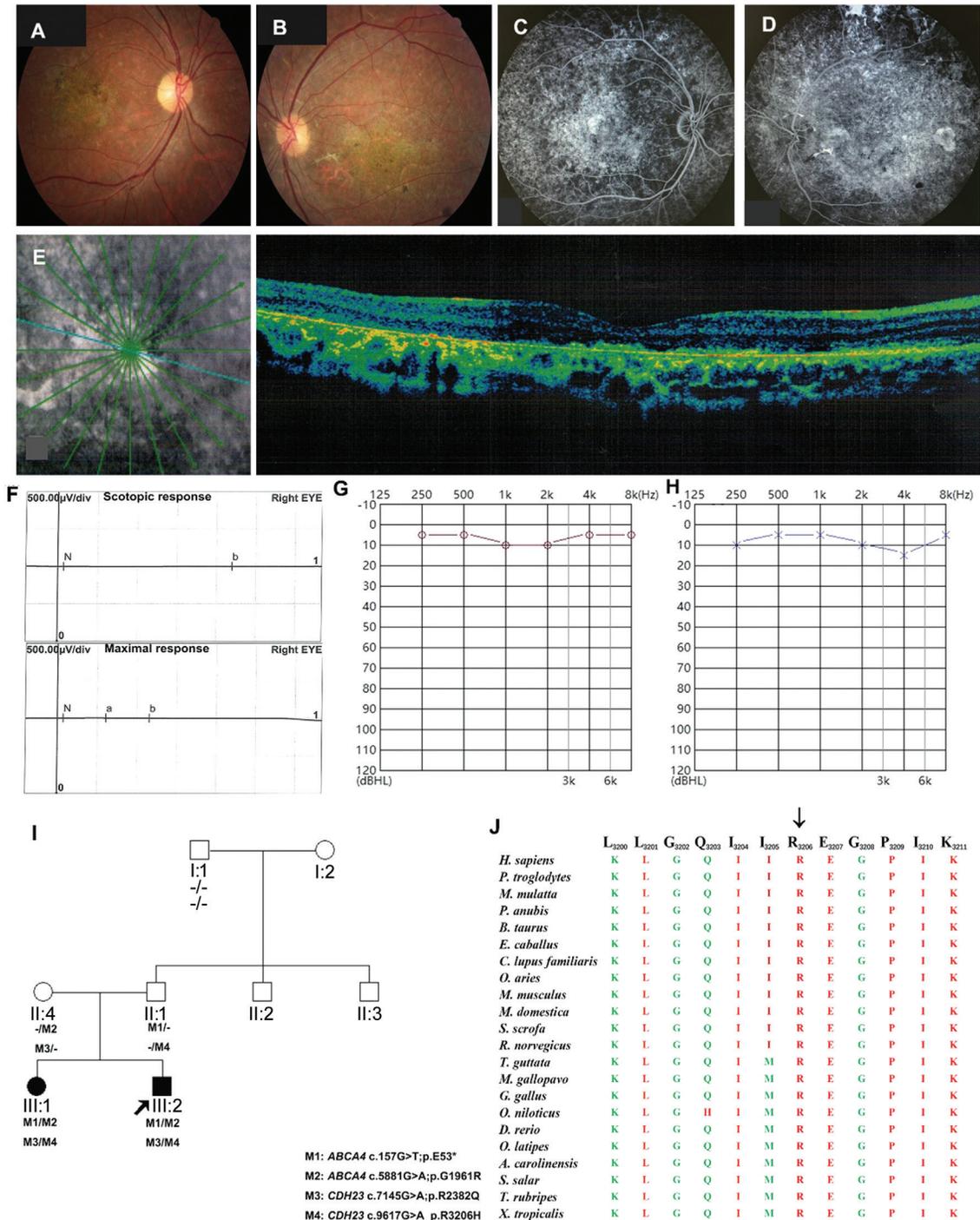


Figure 2 Clinical characteristics of the patient RP02-III:2 A, B: Fundus examination shows bilateral beaten-bronze appearing macular lesions with yellow flecks around the macula, retinal arteriolar attenuation, and widespread RPE atrophy; C, D: FFA presents with hyperfluorescence in the poster pole due to RPE atrophy and hypofluorescent dots corresponding to the yellow flecks observed in the fundus examination; E: OCT images show thinning of the retina in the macula; F: Full-field ERG shows that both cone and rod responses were markedly reduced; G, H: Pure tone audiometry examination showed the patient's normal hearing ability; I: Pedigree of family RP02; J: Multiple orthologous sequence alignment of CDH23 around the R3206 amino acid in 22 species.

4E). Full-field ERG showed markedly reduced rod responses (Figure 4F). Pure tone audiometry examination showed that the patient has moderate hearing impairment (Figure 4G, 4H). Genetic study showed the proband was harboring compound heterozygous variants c.6986C>A (p.P2329H) and c.4758+3A>G in USH2A and a homozygous variant c.2530C>T (p.Q844*)

in EYS (Figure 4I). The missense substitution c.6986C>A (p.P2329H) in USH2A gene was first detected in this study. It affected a conserved proline and was predicted to be disease-causing. The variant p.P2329H localized to the 9th fibronectin type III of usherin^[31], which was highly conserved (Figure 4J). The variant p.Q844* of EYS gene was novel. The EYS gene

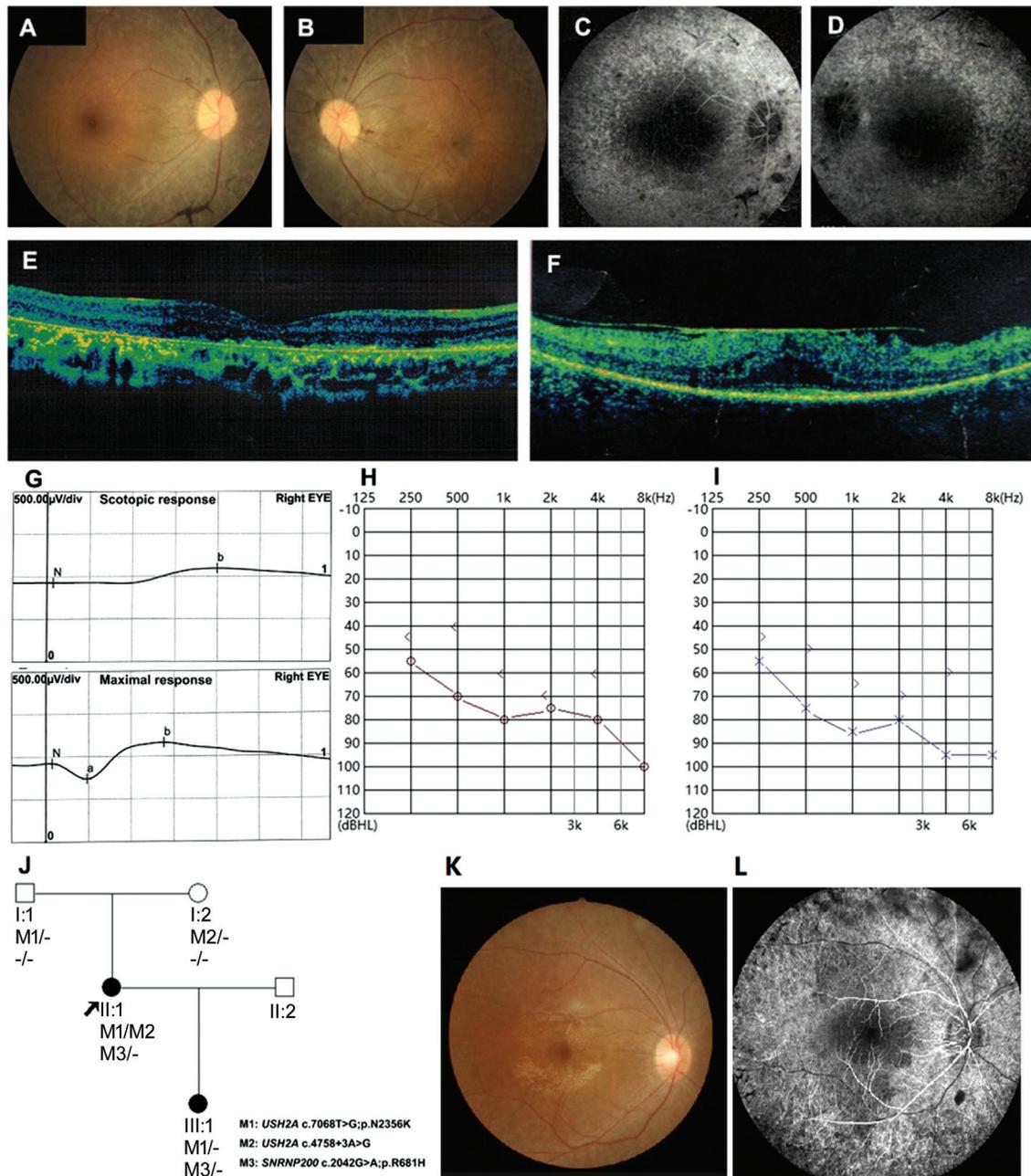


Figure 3 Clinical characteristics of the patient RP03-II:1 A, B: Fundus examination of the patient RP03-II:1 shows bilateral retinal arteriolar attenuation, widespread RPE atrophy, pigment deposition, and pale optic disc; it also shows a macular epiretinal membrane in her left eye; C, D: FFA of the proband’s both eyes RP03-II:1 demonstrates scattered hyperfluorescent RPE; E, F: OCT shows loss of the inner-segment ellipsoid and outer nuclear layer in the retina, with a macular epiretinal membrane in her left eye; G: Full-field ERG showed markedly reduced rod responses; H, I: Pure tone audiometry examination showed that the patient has serious hearing impairment; J: Pedigree of family RP03; K: Fundus examination of the proband’s daughter shows slight RP changes such as retinal arteriolar attenuation and RPE atrophy; L: FFA of the proband’s daughter shows scattered hyperfluorescent RPE in the poster pole.

encodes the eyes shut/spacemaker (*Drosophila*) homolog protein, involving in visual perception^[32]. The variants in *EYS* are recognized as major causes for arRP (RetNet; <http://www.sph.uth.tmc.edu/Retnet/>). Considering the clinical manifestation such as hearing impairment and genetic identification, the patient was affected with an overlapping manifestation of both autosomal recessive Usher syndrome and arRP.

DISCUSSION

In this study, apart from the two disease-causing alleles in *ABCA4*, patients RP01-II:1 and RP02-III:2 were detected to harbor two other disease-causing alleles in *USH2A* and *CDH23*, respectively, leading to a combined autosomal recessive STGD1 disease and arRP. In addition to the two disease-causing alleles in *USH2A*, patient RP03-II:1 was found to carry a heterozygous disease-causing variant in *SNRNP200*,

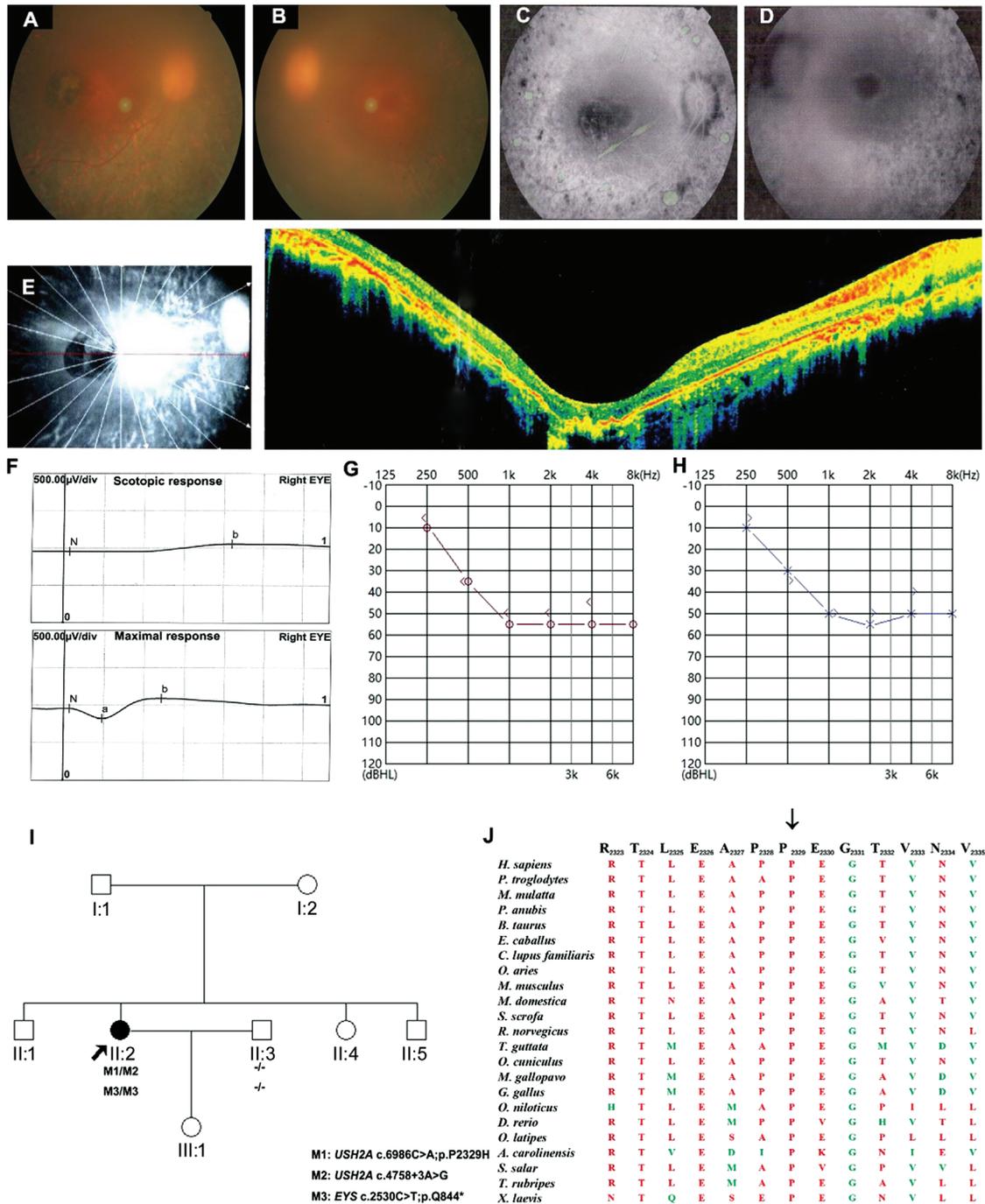


Figure 4 Clinical characteristics of the patient RP04-II:2 A, B: Fundus examination the patient RP04-II:2 shows bilateral widespread RPE atrophy and retinal arteriolar attenuation, which are not distinguished clearly because of cataract lesions in her both eyes; C, D: FFA of the patient RP04-II:2 demonstrates scattered hyperfluorescent RPE in her both eyes; E: OCT the patient RP04-II:2 shows thinning of the outer retina in her left eye; F: Full-field ERG showed markedly reduced rod responses; G, H: Pure tone audiometry examination showed that the patient has moderate hearing impairment; I: Pedigree of family RP04; J: Multiple orthologous sequence alignment of *USH2A* around the P2329 amino acid in 23 species.

which was passed to her affected daughter, resulting in a combined autosomal recessive Usher syndrome and adRP in the proband; patient RP04-II:2 was found to harbor the other homozygous variant in *EYS*, resulting in a combined autosomal recessive Usher syndrome and arRP at the same time, which implies that a comprehensive genetic diagnosis is very important.

IRDs are uncommon, and the co-existence of RP and diseases in other organs in a person is not extraordinary; however, the likelihood of affecting with more than one hereditary disease in the same organ is rare. The incidence of RP is approximately 1:4000^[9] and that of STGD1 is approximately 1:10 000^[5-6], making the simultaneous prevalence of both STGD1 and RP highly improbable (about 1 in 40 million). However, in this

study two families were affected with combined autosomal recessive STGD1 disease (due to *ABCA4* variants) and arRP (due to *USH2A* and *CDH23* variants respectively) simultaneously. The spectrum of *ABCA4*-associated disease consists of a large, heterogeneous group of recessive retinal dystrophies, including STGD1 and RP *etc*^[24,33]. According to the latest data from the Human Gene Mutation Database, there are 104 variants in *ABCA4* gene were responsible for STGD1 phenotype and 31 variants for RP phenotype. The probands of RP01 and RP02 demonstrated the classical manifestation of STGD1 caused by *ABCA4*, while they also displayed nyctalopia and rod abnormalities in full-field ERG which was not consistent of STGD1, but RP.

The *USH2A* gene, encoding usherin, causes autosomal recessive Usher syndrome and arRP; however, it remains unknown why some variants in *USH2A* result in Usher syndrome type IIa while others to non-syndromic RP^[34]. The function of the usherin protein is the maintenance of photoreceptor cells in the retina^[35]. From our previous studies (data not shown) and from manifestation of family RP03 and RP04 in this study, most patients carried *USH2A* variants demonstrated hearing disability after their 40s, although the patient RP01-II:1 did not complaint of hearing loss, it might happen in the future. Mutational defects in *CDH23*, encoding cadherin-23 (*USH1D* protein), result in congenital deaf and vestibular dysfunction, and develop RP in the first decade of life generally^[36-37]. Data from previous studies showed that missense variants in *CDH23* produce a less severe phenotype, both in families with Usher syndrome type I and with recessive non-syndromic deafness (RNSD), whereas nonsense, splice-site, or frameshift variants cause a more severe Usher syndrome type I phenotype^[36,38]. Previously, Besnard *et al*^[39] reported a patient affected with Usher syndrome type II carrying compound heterozygous variants in the *CDH23* gene, furthermore, genes associated with a certain syndromic disorder can also be associated with non-syndromic IRD. Besides, in some cases, the different phenotypes can result from the same variant exactly^[40], which was probably due to environment differences or genetic modifiers^[36,38], consisting with the fact that IRDs are clinically and genetically heterogeneous. The clinical feature of patient RP02-III:2 support our assumption that *CDH23* is a RP-related gene, indicating. Further investigation was needed to confirm this assumption in the future, at present he has no hearing loss, but may suffer from it when he gets older, and his clinical molecular diagnosis would be refined the RP and congenital deafness caused by variants in *CDH23*.

RP affects approximately 1 in 3500 individuals worldwide, making the prevalence of RP overlay with RP higher than that of STGD1 overlay with RP. From a previous report,

approximately 12% to 25% of non-syndromic RP patients and 50% to 75% of Usher syndrome patients carry variants in *USH2A*, making it one of the most important IRD genes in these populations^[41]. Variants in the *EYS* gene account for 5-18% of arRP patients in different populations^[42-44], and the prevalence of *SNRNP200*-associated RP is at least 4.2%^[30]. *USH2A*, *SNRNP200*, and *EYS* are all common disease-causing genes, making the occurrence of two disease-causing genes in one patient not very rare. However, definite phenotype-genotype correlations amongst even the most frequent variants are rarely understood, it is difficult to discern which phenotype was result from a specific genetic defect.

IRDs are great genetically and clinically heterogeneous, making traditional methods such as individual gene screening, genotyping microarray, or arrayed primer extension chip difficult to perform and unlikely to reveal the full mutational spectrum in these patients. Targeted re-sequencing offers an effective, time- and cost-saving tool for molecular diagnosis of these patients. Phenotypes of patients affected with IRDs sometimes vary a lot, even within the same family. Neither targeted genetic screening nor clinical examination alone can offer clinicians complete and comprehensive information for diagnosis; instead, a combination of molecular diagnosis and clinical manifestations help arrive at an accurate and comprehensive clinical diagnosis can better guide disease management and family planning for these patients^[1,13].

There is one point need to be noted. It has been reported that non-pathogenic *PRPH2/ROM1* variants, when present together, result in digenic RP, *ROM1* may serve as a disease modifier by contributing to the large variability in *PRPH2*-associated disease phenotypes^[45]. In the present study, whether *USH2A* interacts with *SNRNP200* or *EYS* to contribute to milder or severer RP phenotype? At present we could not differentiate *USH2A*- from *SNRNP200*- or *EYS*- associated RP phenotypes in a certain patient. All these need to be further investigated in the future. In summary, four Chinese IRD patients have been identified as harboring disease-causing variants in two different disease-causing genes, leading to a complex and overlapping disease phenotype.

In the study, we emphasize the significance of genotype-phenotype correlation and comprehensive genetic screening in IRDs patients, increasing our knowledge of the complex molecular diagnosis of IRDs, which aids in the family planning and gene-based therapy for patients in the future.

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