Targeted next-generation sequencing extends the phenotypic and mutational spectrums for EYS mutations

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Purpose: We aim to determine genetic lesions with a phenotypic correlation in four Chinese families with autosomal recessive retinitis pigmentosa (RP).

Methods: Medical histories were carefully reviewed. All patients received comprehensive ophthalmic evaluations. The next-generation sequencing (NGS) approach targeting a panel of 205 retinal disease–relevant genes and 15 candidate genes was selectively performed on probands from the four recruited families for mutation detection. Online predictive software and crystal structure modeling were also applied to test the potential pathogenic effects of identified mutations.

Results: Of the four families, two were diagnosed with RP sino pigmento (RPSP). Patients with RPSP claimed to have earlier RP age of onset but slower disease progression. Five mutations in the eyes shut homolog (EYS) gene, involving two novel (c.7228+1G:A and c.9248G:A) and three recurrent mutations (c.4957dupA, c.6416G>A and c.6557G>A), were found as RP causative in the four families. The missense variant c.5093T>C was determined to be a variant of unknown significance (VUS) due to the variant’s colocalization in the same allele with the reported pathogenic mutation c.6416G>A. The two novel variants were further confirmed absent in 100 unrelated healthy controls. Online predictive software indicated potential pathogenicity of the three missense mutations. Further, crystal structural modeling suggested generation of two abnormal hydrogen bonds by the missense mutation p.G2186E (c.6557G>A) and elongation of its neighboring β-sheet induced by p.G3083D (c.9248G>A), which could alter the tertiary structure of the eys protein and thus interrupt its physicochemical properties.

Conclusions: Taken together, with the targeted NGS approach, we reveal novel EYS mutations and prove the efficiency of targeted NGS in the genetic diagnoses of RP. We also first report the correlation between EYS mutations and RPSP. The genotypic-phenotypic relationship in all Chinese patients carrying mutations in the EYS gene were also reviewed and summarized.

Inherited retinal dystrophies (IRDs) are a group of disorders characterized by progressive dysfunction of photoreceptors and vision loss. Despite clinical and genetic heterogeneities, IRDs are mostly monogenic diseases. Retinitis pigmentosa (RP; MIM: 268,000), the most common form of IRDs, is characterized by primary dysfunctions of the rod photoreceptors followed by degeneration of the RPE and cone photoreceptors [1]. Clinical hallmarks of RP include night blindness, progressive visual field (VF) constrictions, and final loss of central vision. In genetic aspects, RP is a monogenic disease that can be inherited via all three Mendelian inheritance modes, including autosomal dominant, autosomal recessive, and X-linked patterns [2]. Digenic traits have also been reported [3,4]. To date, 82 RP causative genes and 89 RP-associated loci have been reported. The remarkable genetic heterogeneity calls for an efficient and high throughput technique to assist the molecular diagnosis of RP. Next-generation sequencing (NGS) is capable of detecting mutations with high efficiency and precision, and has therefore been considered the most efficient approach for mutation screening [5,6].

The eyes shut homolog (EYS; OMIM: 612424) gene, located on chromosome 6p12 and spanning 2 Mb, is the largest eye-specific gene identified by far [7,8]. The protein eyes shut homolog, the protein encoded by the EYS gene, is a 3,165-amino-acid protein that contains five laminin G-like domains and 27 epidermal growth factor (EGF)-like domains. This protein shows exclusive expression in the outer segments of photoreceptors and functions in the visual perception process [8]. Mutations in the EYS gene are among the most prominent causes of RP and even some other types of IRDs [9-11]. Herein, with a targeted NGS approach, we reveal five EYS mutations as disease causative in four Chinese
families with RP. The genotypic-phenotypic correlations in all Chinese patients carrying mutations in the EYS gene are reviewed and discussed.

METHODS

Participants and clinical evaluations: This study, designed in accordance with the tenets of the Declaration of Helsinki and adhered to the ARVO statement on human subjects, was approved by the Ethics Committee on Human Research of Nanjing Medical University. Written informed consent was obtained from all participants before participation. Four patients with IRDs and seven unaffected family members from four individual families were recruited from the First Affiliated Hospital of Nanjing Medical University (Figure 1A). All participants received routine ophthalmic examinations, including assessments of best-corrected visual acuity (BCVA; Snellen), slit-lamp examinations, funduscope, and VF measurements. Comprehensive ophthalmic tests, including an electoretinogram (ERG) test, optical coherence tomography (OCT) examination, and fundus fluorescein angiography (FFA), were performed on the two included patients. Additionally, 100 unrelated Chinese controls with neither major ocular diseases as demonstrated with routine ophthalmic examinations nor family history of any type of IRD were included in the study for prevalence tests. Peripheral blood samples were collected in EDTA tubes. DNA extraction from leukocytes was performed using a QIAmp DNA blood kit (Qiagen, Valencia, CA) according to the manufacturer’s protocols.

Targeted NGS approach, bioinformatics analyses, and Sanger sequencing: A targeted NGS approach was selectively conducted on probands from the four individual families, ARRP07-II:1, ARRP08-II:2, ARRP09-II:1 and ARRP10-II:3, using a custom-designed in-solution capture array targeting 205 reported retinal disease-associated genes and 15 candidate genes. Candidate genes were included based on publications and our previous work. Details of the 220 selected genes are provided in Appendix 1. This capture array targeted the coding sequences, 100 bp flanking regions, and 5’- and 3’-untranslated regions (UTR) of each selected gene. A total of 40 samples, including the four probands’ samples, were multiplexed in the Illumina HiSeq2000 platform (Illumina, Inc., San Diego, CA). Library preparation, qualification, and NGS on the Illumina HiSeq2000 platform were performed as described previously [12-16]. Briefly, 6 μg of the patients’ DNA samples was randomly fragmented into 250 to 300 bp segments. The fragments were then ligated with adaptors and amplified via ligation-mediated polymerase chain reaction (LM-PCR) [17]. DNA was then purified and hybridized for enrichment. Quantitative PCR was then performed on non-captured and captured LM-PCR products to estimate the magnitude of enrichment. Sequence reads were then aligned to the reference human genome 19 (hg19) for annotations of single nucleotide variations (SNVs) together with insertions and deletions (indel). The annotated variants were then filtered against six single nucleotide polymorphism (SNP) databases, including dbSNP144, HapMap project, 1000 Genome Project, YH database, Exome Variant Server (EV), and Exome Aggregation Consortium (EXAC). Sanger sequencing was used for mutation verification and prevalence testing according to a previously defined protocol [18]. Primer information is provided in Appendix 2.

In silico analyses: SWISS-MODEL online server was applied to construct the crystal structural models of the wild-type and mutant proteins [19,20]. Predicted structures were visualized and compared using PyMol software (version 1.5). Potential pathogenicity of the identified mutations was evaluated using three types of online predictive software, including Sorting Intolerant From Tolerant (SIFT) [21], polymorphism pheno-typing v2 (PolyPhene-2) [22], and Protein Variation Effect Analyzer (PROVEAN) [23].

RESULTS

Clinical manifestations: All four probands presented typical RP symptoms, including initial nyctalopia, constricted VF, and decreased visual acuities. The RP age of onset and disease progression varied greatly among different patients. ARRP07-II:1, a 33-year-old female patient, claimed to have had night blindness since age 8, the youngest disease age of onset among all investigated patients. However, her disease progressed so slowly that she did not notice VF constriction until age 24. Her current BCVA was 20/50 for the right eye and 20/40 for the left eye with her current VF less than 15° for both eyes. Patient ARRP09-II:1, aged 56, showed nyctalopia since age 20, and her disease progressed slowly. Her BCVA was 20/50 for both eyes at her last visit to our hospital. Patients ARRP08-II:2 and ARRP10-II:3 showed late RP onset but fast disease progression. Patient ARRP08-II:2, aged 40, had nyctalopia at age 35. His current BCVA was 20/40 for both eyes, and his current VF was less than 30°. ARRP10-II:3, a female patient aged 41, reported initial symptoms at age 32. Her BCVA was 20/40 for both eyes.

Ophthalmic examinations indicated typical RP fundus, including optic disc pallor, attenuated retinal arterioles, and bone spicule-like pigments in the mid-peripheral retina of patients ARRP08-II:2 and ARRP10-II:3 (Figure 2B,D). No pigment deposit was observed in the fundus of patients ARRP07-II:1 or ARRP09-II:1, which helped to modify
The clinical diagnoses of patients were consistent with RP with pigmentary alterations (RPSP; Figure 2A,C). OCT presentations showed a thickened inner/outer segment (IS/OS) layer with a preserved macular structure in all four recruited patients (Figure 2F–I). Significant decreases in ERG presentations were also observed in all included patients. No patient reported systemic defects.

**Genetic findings:** Targeted NGS was selectively performed on patients ARRP07-II:1, ARRP08-II:2, ARRP09-II:1, and ARRP10-II:3. The NGS results are detailed in Appendix 3. Briefly, the average coverage and depth of the targeted region reached 94.68% and 60.34-fold, respectively. A total of 8,142 variants covering 7,236 SNPs and 906 indels were revealed.
with the initial NGS approach in all four tested patients. The detailed process for filtration and validation is demonstrated in Table 1. After comprehensive genetic analyses, we confirmed six variations in the EYS gene (NM_001142800; NP_001136272) that segregated the disease phenotypes within the four families. A homozygous recurrent missense mutation, c.6557G>A, was found carried by patient ARRP07-II:1 (Figure 1A,B) [10]. This mutation would lead to the substitution from the neutral glycine to the acidic glutamic acid at residue 2186, which was located in the second Laminin G-like domain of the protein eyes shut homolog (Figure 3). All three types of online predictive software suggested the potential deleterious effect of this mutation (Table 2). Crystal structures of the wild-type and mutant eys proteins carrying p.G2186E were built based on template 3poy.1A as indicated previously [24]. This mutation was predicted to cause the generation of two hydrogen bonds between the mutated glutamic acid at residue 2186 and arginine at residue 2306 and threonine at residue 2181, respectively (Figure 3B,C).

Biallelic EYS variants, c.[5093T>C; 6416G>A];[9248G>A], were found to be carried by patient ARRP08-II:2 (Figure 1A,C–E). The missense variant c.9248G>A was novel, while c.5093T>C was determined as a variant of unknown significance (VUS) due to its colocalization in the same allele with the reported pathogenic mutation c.6416G>A. The missense variant c.9248G>A would induce a change from the neutral glycine to the acidic aspartic acid at residue 3083 (p.G3083D; Figure 1A,E and Figure 3). The crystal structure of the mutant protein with p.G3083D indicated shortening of the neighboring β-sheet, which could alter its tertiary structure and physicochemical properties.
| Items                                      | ARRP07 | ARRP08 | ARRP09 | ARRP10 |
|-------------------------------------------|--------|--------|--------|--------|
| SNVs/Indels                               |        |        |        |        |
| Initial variants                          | 1353   | 159    | 1289   | 147    |
| Excluded variants found in SNP databases  | 1312   | 149    | 135    | 128    |
| Remaining variants                        | 41     | 9      | 52     | 12     |
| Excluded non-coding variants              | 34     | 9      | 43     | 12     |
| Remaining variants                        | 7      | 0      | 9      | 0      |
| Excluded false positive variants          | 7      | 0      | 9      | 0      |
| Remaining variants                        | 7      | 0      | 9      | 0      |
| Excluded variants failed intra-familial cosegregation analysis | 7      | 0      | 9      | 0      |
| Remaining variants                        | 1      | 0      | 3      | 0      |
| Excluded variants failed intra-familial cosegregation analysis | 1      | 0      | 2      | 0      |

Abbreviations: SNVs: single nucleotide variations; Indels: insertions and deletions.
(Figure 3D,E). The recurrent mutation c.6416G>A would lead to an amino acid change from the hydrophilic cysteine to the hydrophobic tyrosine at residue 2139 (p.C2139Y). Our previous work revealed the generation of a novel hydrogen bond and vanishing of the original twist led by this mutation [24]. The VUS c.5093T>C would lead to the substitution from the hydrophobic isoleucine to the hydrophilic threonine at residue 1698 (p.I1698T).

Similar to patient ARRP08-II:2, biallelic EYS mutations, c.[6557G>A];[7228+1G>A], were found to be carried by patient ARRP09-II:1. The missense c.6557G>A was the same recurrent mutation carried by patient ARRP07-II:1. The novel splice-site variation c.7228+1G>A was predicted to abolish the regular splice donor site and generate a truncated protein p.A2410Gfs*4 or cause nonsense-mediated mRNA decay (NMD). This variation was found to be shared by patient ARRP10-II:3, who carried biallelic heterozygous c.[4957dupA];[7228+1G>A]. The first variant was a reported frameshift variation that might generate a truncated protein p.S1653Kfs*2 or cause NMD. The two novel variations, c.7228+1G>A and c.9248G>A, were further confirmed absent in the 100 unrelated healthy controls.

**DISCUSSION**

Mutations in the EYS gene play a prominent causative role in RP etiology, and their involvement in cone-rod dystrophy (CRD) has also been revealed in recent studies [25,26]. EYS mutations that give rise to a panel of phenotypes with their inter- and intrafamilial phenotypic diversities been reported [8,24]. Therefore, a clear insight into the genotypic and phenotypic spectrums for EYS mutations will help with better clinical management. In the present study, two novel and three recurrent EYS mutations, together with one VUS, were found to be RP causative for four individual families. We also for the first time report the correlation between EYS mutation and RPSP. The genotype and phenotype correlations in all Chinese patients carrying EYS mutations were reviewed and are summarized in Table 3 [24,27–29].

Two sporadic patients, including a 33-year-old female with the age of onset of 8 and a 56-year-old female with the age of onset of 20, were finalized with a clinical diagnosis

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**Figure 3.** EYS variations identified in the present study. **A:** Schematic representation of the linear location of the identified EYS variants in the context of the genome (upper) and the protein (below). **B–E:** Crystal structural models of the wild-type (B and D) and mutant protein eyes shut homolog (C and E).
### Table 2. Characteristics of identified EYS mutations.

| Family ID | Disease | Variation | Genomic position | Nucleotide | Amino Acid | Type       | Status | Exon /Intron | Bioinformatics Analysis | HGMD/Novel |
|-----------|---------|-----------|------------------|------------|------------|------------|--------|--------------|-------------------------|------------|
| ARRP07    | RPSP    | c.6557G>A | 6: 64,791,763    | p.G2186E   | missense   | Hom        | E32    | D (0.03)     | PD (0.68)              | CM103598   |
| ARRP08    | RP      | c.5093T>C | 6: 65,300,667    | p.I1698T   | missense   | Het        | E26    | DA (0)      | B (0.082)              | VUS        |
|           |         | c.9248G>A | 6: 64,430,679    | p.G3083D   | missense   | Het        | E43    | D (0.02)    | DA (0.979)              | Novel      |
| ARRP09    | RPSP    | c.6557G>A | 6: 64,791,763    | p.G2186E   | missense   | Het        | E31    | DA (0)      | PD (0.797)              | CM102730   |
| ARRP10    | RP      | c.7228+1G>A| 6: 64,574,078   | p.A2410Gfs*4| splice site| Het        | I36–37 | NA          | NA                      | Novel      |
|           |         | c.7228+1G>A| 6: 64,574,078   | p.A2410Gfs*4| splice site| Het        | I36–37 | NA          | NA                      | Novel      |
|           |         | c.4957dupA| 6: 65,300,802    | p.S1653Kfs*2| frameshift | Het        | E26    | NA          | NA                      | Novel      |

**Abbreviations:** RPSP: retinitis pigmentosa sino pigmento; RP: retinitis pigmentosa; Hom: homozygous; Het: heterozygous; DA: damaging; NA: not available; PD: probably damaging; B: benign; DE: deleterious; N: neutral; HGMD: the Human Gene Mutation Database; VUS: variant of unknown significance.
| Mutation | Type | Nucleotide | Amino Acid | Patient ID | Disease | Age (year)/Sex | Onset Age (year) | Clinical presentation | Reference |
|----------|------|------------|------------|------------|---------|----------------|------------------|----------------------|-----------|
| 1*       | -    | p.E1836* (Hom) | II:1       | RP         | NA/F   | 20             | 20/400          | HM                   | Diminished pallor | [28]      |
| 1*       | -    | p.E1836* (Hom) | II:2       | RP         | 40/M   | 15             | 20/400          | 20/400 Diffuse       | Mild pallor        | [28]      |
| 1*       | -    | p.E1836* (Hom) | II:5       | RP         | NA/F   | 20             | 20/400          | HM                   | Diminished pallor | [28]      |
| 1*       | c.[8012T>A];[704G>A] | p.[L2671*];[W235*] | F35–1      | IRDs      | 43/M   | 13             | 20/100          | 20/100 Diffuse       | Diminished pallor | [29]      |
| 2°      | c.[490C>T];[6416G>A] | p.[R614*];[C2139Y] | ARRP05-II:3 | RP    | 40/M  | 22             | FC             | FC Diffuse Waxy      | Diminished pallor | [24]      |
| 2°      | c.[490C>T];[6416G>A] | p.[R614*];[C2139Y] | ARRP05-II:5 | RP    | 37/M  | 20             | HM             | HM                   | Diminished         | [24]      |
| 2°      | c.[490C>T];[6416G>A] | p.[R614*];[C2139Y] | ARRP05-II:7 | RP    | 32/M  | 25             | 20/200          | 20/200 Diffuse       | Diminished pallor | [24]      |
| 2°      | c.[7919G>A];[8861T>C] | p.[W2640*];[F2954S] | ARRP06-II:2 | RP    | 54/M  | 18             | LP             | LP Diffuse Waxy      | Diminished pal.... | [24]      |
| 2°      | c.[7919G>A];[8861T>C] | p.[W2640*];[F2954S] | ARRP06-II:4 | RP    | 52/M  | 35             | LP             | LP Diffuse Waxy      | Diminished pal.... | [24]      |
| 2°      | c.[7919G>A];[8861T>C] | p.[W2640*];[F2954S] | ARRP06-II:8 | RP    | 49/F  | 32             | 20/25           | 20/25 Diffuse Waxy   | Reduced Constricted | [24]      |
| 2°      | c.[6 5 5 7 G > A ] ; [9186_9187delCA] | p.[G2186E];[NA] | W127–1 | IRDs | 48/F | 20 | 20/100 | 20/500 | Diffuse | Diminished | [29] |
| 2°      | c.[6416G>A];[8150delA] | p.[C2139Y];[NA] | W286–1 | IRDs | 43/M | 20 | FC | 20/400 | - | - | - | [29] |
| 2°      | c.[2510G>T];[8107G>T] | p.[C837F];[E2703*] | F11–1 | IRDs | 48/M | 41 | 20/50 | 20/50 | - | - | - | [29] |
| 2°      | c. [ 7 4 9 2 G > C ] ; [8244_8245insT] | p.[A2498P];[NA] | W82–1 | IRDs | 43/F | 22 | 20/40 | 20/40 | - | - | - | [29] |
| 2°      | c.[6416G>A];[8392delG] | p.[C2139Y];[NA] | W86–1 | IRDs | 25/M | 15 | 20/32 | 20/32 | - | - | - | [29] |
| 2°      | c.[6416G>A];[6557G>A] | p.[C2139Y];[G2186E] | No.10-II:1 | RP | 49/F | 42 | 20/30 | 20/30 | Diffuse | Diminished pallor | [27] |
| 2°      | c.[6416G>A];[6557G>A] | p.[C2139Y];[G2186E] | No.10-II:3 | RP | 45/M | 36 | 20/50 | 20/40 | Diffuse | Diminished pallor | [27] |
| 2°      | c.[6416G>A];[6557G>A] | p.[C2139Y];[G2186E] | No.10-II:4 | RP | 35/F | 20 | 20/30 | 20/30 | Diffuse | Diminished pallor | [27] |
| 2°      | c.[9368A>C];[6416G>A] | p.[N3123T];[C2139Y] | W5–1 | IRDs | 34/M | 14 | 20/40 | 20/40 | - | - | - | [29] |
| Mutation Type | Nucleotide | Amino Acid | Patient ID | Disease | Age (year)/Sex | Onset Age (year) | Clinical presentation | Reference |
|---------------|------------|------------|------------|---------|----------------|------------------|----------------------|-----------|
| 3<sup>a</sup> | c.7492G>C (Hom) | p.A2498P (Hom) | W159–1 | IRDs | 34/M | 20 | 20/40 | 20/50 | - | - | - | [29] |
| 3<sup>a</sup> | c.[7609G>A];[7949C>T] | p.[A2537T];[S2650F] | W171–1 | IRDs | 37/F | 20 | FC | FC | - | - | - | [29] |
| 3<sup>a</sup> | c.[3489T>A];[2644T>C] | p.[N1163K];[F882L] | W13–1 | IRDs | 63/M | 41 | 20/125 | 20/100 | - | - | - | [29] |
| 3<sup>a</sup> | c.6416G>A (Hom) | p.C2139Y (Hom) | W75–1 | IRDs | 31/M | 20 | 20/100 | 20/100 | - | - | - | [29] |

**Abbreviations:** * loss of function & loss of function alleles; # loss of function & missense alleles; $ missense & missense alleles; RP: retinitis pigmentosa; IRDs: inherited retinal degenerations; Hom: homozygous; BCVA: best corrected visual acuity; O.D.: right eye; O.S.: left eye; FC: finger count; HM: hand movement; LP: light perception; ERG: electroretinography; VF: visual field.
of RPSP based on comprehensive ophthalmic examinations. The recurrent mutation, *EYS* p.G2186E, a mutational hotspot among multiple ethnic groups [10,27,30], was found to be carried by both patients. Crystal structural analysis suggests the generation of two abnormal hydrogen bonds caused by this mutation, which alters the tertiary structure of the protein and interrupts its physicochemical properties. In previous studies, the RP age of onset of patients carrying p.G2186E range from 14- to 62 years-old, while one patient in this study carrying a homozygous p.G2186E mutation claims to have had night blindness at age 8, younger than all reported cases. This mutation has been reported in two Chinese families, including a family with autosomal recessive retinal pigmentosa (arRP) and a sporadic case (Table 3) [27,29]. The age of onset ranged from 20 to 42 and demonstrated remarkable inter-familial diversity. Diffuse pigmentations are detected in all patients from the family with arRP, while no pigmentation was found in the two cases in our study. The elder patient also carries a novel heterozygous *EYS* mutation c.7228+1 G>A, a splice-site variant that may eliminate the regular splice site and generate a truncated protein p.A2410Gfs*4 or cause NMD. This variant was also found to be carried by another patient with typical RP, implying correlated phenotypic diversity. To the best of our knowledge, this is the first time that an *EYS* mutation was found to be correlated with RPSP.

Another hotspot *EYS* mutation, p.C2139Y located in the EGF-like domain of the eys protein, was observed in a 40-year-old male patient with RP [26,27,29]. This patient developed RP at age 35, and the disease progressed quickly since its development. According to our summary, this is most common *EYS* mutation among the Chinese population with age of onset ranging from 14- to 42 years-old (Table 3). Our group has recently reported a Chinese family carrying this mutation with similar late onset and rapid progress [24]. This patient also carries a novel heterozygous *EYS* mutation, p.G3083D, which has been found to induce the elongation of its neighboring β-sheet, and a missense VUS c.5093T>C colocalized in the same allele with the reported pathogenic mutation p.C2139Y. Another recurrent mutation, the frameshift c.4957dupA, was found to be carried by patient ARRP10-II:3, the same patient who carries c.7228+1G>A. This mutation has not been previously reported in the Chinese population but is a hotspot in the Japanese population [30,31].

In summary, with a targeted NGS approach, we reveal five *EYS* mutations as disease causative for four Chinese RP families with the genotype–phenotype correlations annotated. We also, for the first time, report the implication of *EYS* mutations in the etiology of RPSP, which extends the phenotypic spectrum for *EYS*-associated retinopathy. Genetic assessments would help with better clinical management of patients with IRDs. Taken together, our study provides novel insights into the etiology of RP, which are important for clinicians to obtain better genetic diagnosis, prognosis, and personalized treatment for patients with RP. However, the specific pathogenic mechanism underlying the phenotypic variability correlated with *EYS* mutations is currently under debate. Future investigations are still warranted.

APPENDIX 1. TARGETED GENES CAPTURED BY THE CUSTOM-DESIGNED IN-SOLUTION CAPTURE ARRAY

To access the data, click or select the words “Appendix 1.”

APPENDIX 2. PRIMER INFORMATION MUTATION VERIFICATION.

Base pair: bp To access the data, click or select the words “Appendix 2.”

APPENDIX 3. OVERVIEW OF DATA PRODUCTION.

To access the data, click or select the words “Appendix 3.”

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