Supplementary Material - A novel statistical method for interpreting the pathogenicity of rare variants

Supplementary Methods:

Derivation of the Binomial test1

1) For an autosomal recessive (AR) disease gene gr:

Let “A” denote all the wild type chromosomes for gene gr, and “a” denote all the chromosomes carrying pathogenic variants in gene gr.

Let $p =$ the sum of the frequency of all the wild type chromosomes (i.e. A) for the gene $g_r$ in population.

$q =$ the sum of the frequency of all the chromosomes carrying pathogenic variants (i.e. a) in gene $g_r$ in population,

$Q =$ the disease frequency due to the variants in gene $g_r$ in population.

According to the Hardy-Weinberg Equation:

$$p + q = 1$$
$$\Rightarrow (p + q)^2 = 1$$

Hence, $p^2 + 2*p*q + q^2 = 1$ \hspace{1cm} (1)

In equation (1),

$p^2$ is the frequency of AA (the two wild type chromosomes are not necessary to be the same, but they both are benign), namely the proportion of normal individuals that do not carry any pathogenic variants in the gene $g_r$.

$2pq$ is the frequency of Aa (a combination of a chromosome with pathogenic variant and a wild type chromosome), namely the proportion of carriers with pathogenic variants in the gene $g_r$.

$q^2$ is the frequency of aa (the two chromosomes are not necessary to be same, but they both carry pathogenic variant), namely the proportion of individuals who carry two chromosomes both with pathogenic variant in the gene $g_r$ and are the patients attributed to variants in the gene $g_r$.

Hence,

$$q^2 = Q$$
$$q = \sqrt{Q}$ \hspace{1cm} (2)
Let the allele frequency (AF) of a single pathogenic variant $a_i$ of the gene $g_r$ in population as $q_i$. Assume each chromosome of the gene $g_r$ in population only carries one pathogenic variant. Because, given the low frequency of pathogenic variant for Mendelian diseases, the chance for each chromosome of gene $g_r$ in population to carry multiple pathogenic variants might be small and could be ignored. Thus, the sum of the frequency of all the pathogenic variants in the gene $g_r \approx$ the sum of the frequency of all the chromosomes carrying pathogenic variant:

$$\sum_i q_i \approx q$$

Given equation (2),

$$\sum_i q_i \approx \sqrt{Q} \quad (3)$$

Let the proportion of the variant $a_i$ among all the pathogenic variants as $q_{i,\text{pathogenic}}$,

$$q_{i,\text{pathogenic}} = \frac{q_i}{\sum_i q_i}$$

Given equation (3),

$$q_{i,\text{pathogenic}} = \frac{q_i}{\sqrt{Q}}$$

Assume that $n$ patients are randomly sampled from patients attributable to variants in gene $g_r$, and each patient carries two pathogenic alleles of the gene $g_r$, thus a total of $2 \times n$ pathogenic alleles were sampled (assuming two variants in each patient are independently sampled. We will simulate the scenario of sampling bias and estimate the effect on the test performance below). Therefore, the expected occurrence count of the variant $a_i$ among the $2 \times n$ pathogenic alleles should follow a Binomial distribution with $N = 2 \times n$ trials and the occurrence frequency (success rate) of

$$q_{i,\text{pathogenic}} = \frac{q_i}{\sqrt{Q}}$$

To test whether a variant $a_k$ of the gene $g_r$ is likely to be pathogenic, we have: The null hypothesis $H_0$ of the test1: The variant $a_k$ is pathogenic, thus its observed occurrence ($x$) in $n$ patients due to the gene $g_r$ follows a Binomial $\left(N = 2n, p = \frac{a_k}{\sqrt{Q}}\right)$ distribution.
The alternative hypothesis H1 of the test: Its observed occurrence (x) does not follow Binomial \( N = 2n, p = \frac{q_k}{\sqrt{Q}} \), and show frequency less than \( \frac{q_k}{\sqrt{Q}} \). Therefore, the variant \( a_k \) is unlikely to be pathogenic. Hence, by conducting a left-tailed Binomial test \( X = x, N = 2n, p = \frac{q_k}{\sqrt{Q}} \), we can test whether H0 should be rejected, namely, whether the observed frequency of \( a_k \) in the \( n \) patients is significantly less than \( \frac{q_k}{\sqrt{Q}} \). The significant p-value of the left-tailed Binomial test would suggest this allele \( a_k \) is unlikely to be pathogenic.

2) For an autosomal dominant (AD) disease gene \( g_d \)

Let “A” denote all the wild type chromosomes for gene \( g_d \), and “a” denote all the chromosomes carrying the pathogenic variants in gene \( g_d \).

Let \( p = \) the sum of the frequency of all the wild type chromosomes (i.e. A) for gene \( g_d \) in population.

\( q = \) the sum of the frequency of all the chromosomes carrying pathogenic variant (i.e. a) in gene \( g_d \) in population.

\( Q = \) the disease frequency due to the variants in gene \( g_d \) in population.

According to the Hardy-Weinberg Equation:

\[ p + q = 1 \quad \text{and} \quad p^2 + 2pq + q^2 = 1 \]

\[ 1 - p^2 = Q \]

Given \( 1 - p = q \), Thus

\[ 1 - \sqrt{1 - Q} = q \] (4)

Let the AF of a single pathogenic variant \( a_i \) of the gene \( g_d \) in population as \( q_i \). Assume each chromosome of gene \( g_d \) in population only carries one pathogenic variant. Because, given the low frequency of pathogenic variants for Mendelian diseases, the chance for each chromosome of gene \( g_d \) in population to carry multiple pathogenic variants might be small and could be ignored. Thus, the sum of the frequency of all the pathogenic variants in the gene \( g_d \) \( \approx \) the sum of the frequency of all the chromosomes carrying pathogenic variant:
\[ \sum_i q_i = q \]

Given equation (4),

\[ \Sigma_i q_i = 1 - \sqrt{1 - Q} \] (5)

Let the frequency of the variant \( a_i \) among all the pathogenic variants as \( q_{i,\text{pathogenic}} \),

\[ q_{i,\text{pathogenic}} = \frac{q_i}{\sum_i q_i} \]

Given equation (5),

\[ q_{i,\text{pathogenic}} = \frac{q_i}{1 - \sqrt{1 - Q}} \]

Assume that \( n \) patients are randomly sampled from patients carrying variants in gene \( g_d \), according to the Hardy-Weinberg Equation (1),

the total number of expected pathogenic alleles \( = \frac{n \times 2 \times p \times q}{2 \times p \times q + q^2} + \frac{n \times q^2 \times 2}{2 \times p \times q + q^2} = n \times (1 + \frac{q}{2-q}) \)

Given equation (4),

\[ = n \times (1 + \frac{1 - \sqrt{1 - Q}}{1 + \sqrt{1 + Q}}) \]

if \( Q \ll 1 \)

the expected number of pathogenic variants in \( n \) patients (with the rare dominant disease) \( \approx n \), similar to each patient only carries one pathogenic allele, thus a total of \( n \) pathogenic alleles were sampled. Therefore, the expected occurrence count of the variant \( a_i \) among the \( n \) pathogenic alleles should follow a Binomial distribution with \( N = n \) trials and the occurrence frequency (success rate) of

\[ q_{i,\text{pathogenic}} = \frac{q_i}{1 - \sqrt{1 - Q}} \]

To test whether a variant \( a_k \) of the gene \( g_d \) is likely to be pathogenic, we have:
The null hypothesis H0 of the test: The variant $a_k$ is pathogenic, thus its observed occurrence ($x$) in the $n$ patients attributed to the gene $g_d$ follows a Binomial \( \left( N = n, p = \frac{q_k}{1 - \sqrt{1 - Q}} \right) \) distribution.

The alternative hypothesis H1 of the test: Its observed occurrence ($x$) does not follow Binomial \( \left( N = n, p = \frac{q_k}{1 - \sqrt{1 - Q}} \right) \), and show frequency less than \( \frac{q_k}{1 - \sqrt{1 - Q}} \). Therefore, the variant $a_k$ is unlikely to be pathogenic.

Hence, by conducting a left-tailed Binomial.test \( \left( X = x, N = n, p = \frac{q_k}{1 - \sqrt{1 - Q}} \right) \), we can test whether H0 should be rejected, namely, whether the observed frequency of $a_k$ in the $n$ patients is significantly less than \( \frac{q_k}{1 - \sqrt{1 - Q}} \). The significant p-value of the left-tailed Binomial.test would suggest this variant $a_k$ is unlikely to be pathogenic.

**Application of the combined test for X-linked genes**

Basically, to test for an X-linked recessive gene, we can count male patients only and apply the AD test. For X-linked dominant gene, the same test for the AD gene can be applied.

**Population stratification**

When population stratification is available for the population and patient datasets, the test should be performed respectively for each ethnicity by matching the ethnicity of patients with that of the population. Specifically, the ethnicity of the population AF should be the same as the ethnicity of the patient cohort.

**The simulation analysis of test power and specificity**

To evaluate the test performance, we simulated patient cohorts with the variant count following a binomial distribution and with a variety of sample sizes. We assumed that the disease is due to variants in gene $g$ with a disease prevalence of $Q$ in population, and a variant, $a_k$, in the gene $g$ has an AF of $q_k$ in population.
The power to detect benign alleles was computed as below

\[
\text{power under AD model} = \sum_{i=0}^{n} f_X(x = i) \times f_Y(y = i)
\]

\[
\text{power under AR model} = \sum_{i=0}^{2n} f_X(x = i) \times f_Y(y = i)
\]

If an allele is benign, its AF in patient cohort should be similar to that in normal population, given it has equal association with normal people and patients.

Thus, to simulate a benign allele \(a_k\),

\(f_X(x = i)\) was set as the probability of observing \((x = i)\) count of \(a_k\) in a patient cohort with a sample size of \(2n\) (AR model) or \(n\) (AD model) given an occurrence rate of \(q_k\) (AR model) or \(2q_k\) (AD model) under a binomial distribution.

For AD model:

\[
f_Y(y = i): \begin{cases} 
1, & \text{if p value of Binomial.test} \left(X = i, N = n, p = \frac{q_k}{1-\sqrt{1-Q}}, \text{left.tail} \right) \leq 0.05 \text{ and } \\
\text{if p value of Binomial.test} \left(X = i, N = n, p = 2 \times q_k, \text{right.tail} \right) > 0.05. \\
0, & \text{if p value of Binomial.test} \left(X = i, N = n, p = \frac{q_k}{1-\sqrt{1-Q}}, \text{left.tail} \right) > 0.05 \text{ or } \\
\text{if p value of Binomial.test} \left(X = i, N = n, p = 2 \times q_k, \text{right.tail} \right) \leq 0.05. 
\end{cases}
\]

For AR model:

\[
f_Y(y = i): \begin{cases} 
1, & \text{if p value of Binomial.test} \left(X = i, N = 2n, p = \frac{q_k}{\sqrt{Q}}, \text{left.tail} \right) \leq 0.05 \text{ and } \\
\text{if p value of Binomial.test} \left(X = i, N = 2n, p = q_k, \text{right.tail} \right) > 0.05. \\
0, & \text{if p value of Binomial.test} \left(X = i, N = 2n, p = \frac{q_k}{\sqrt{Q}}, \text{left.tail} \right) > 0.05 \text{ or } \\
\text{if p value of Binomial.test} \left(X = i, N = 2n, p = q_k, \text{right.tail} \right) \leq 0.05. 
\end{cases}
\]
The specificity to detect pathogenic alleles is computed as below:

\[
\text{specificity under AD model} = \sum_{i=0}^{n} f_X(x = i) \times f_W(w = i)
\]

\[
\text{specificity under AR model} = \sum_{i=0}^{2n} f_X(x = i) \times f_W(w = i)
\]

If an allele \(a_k\) is pathogenic, the expected frequency of \(a_k\) among the pathogenic variants will be \(\frac{q_k}{\sqrt{q}}\) for the AR model and \(\frac{q_k}{1-\sqrt{1-q}}\) for the AD model.

Thus, to simulate a pathogenic allele \(a_k\),
\(f_X(x = i)\) was set as the probability of observing \((x = i)\) count of \(a_k\) in a patient cohort with a sample size of \(2n\) (AR model) or \(n\) (AD model) given an occurrence rate of \(\frac{q_k}{\sqrt{q}}\) (AR model) or \(\frac{q_k}{1-\sqrt{1-q}}\) (AD model) following a binomial distribution.

For AD model:

\(f_W(w = i):\)

\[
\begin{cases} 
1, \text{if p value of Binomial.test} \left( X = i, N = n, p = \frac{q_k}{1-\sqrt{1-q}}, \text{left.tail} \right) > 0.05 \ or \\
\quad \text{if p value of Binomial.test} \left( X = i, N = n, p = 2 \times q_k, \text{right.tail} \right) \leq 0.05. \\
0, \text{if p value of Binomial.test} \left( X = i, N = n, p = \frac{q_k}{1-\sqrt{1-q}}, \text{left.tail} \right) \leq 0.05 \ and \\
\quad \text{if p value of Binomial.test} \left( X = i, N = n, p = 2 \times q_k, \text{right.tail} \right) > 0.05.
\end{cases}
\]

For AR model:

\(f_W(w = i):\)
\[
1, \text{if } p\text{ value of } \text{Binomial.test}\left(X = i, N = 2n, p = \frac{q_k}{\sqrt{Q}}, \text{left. tail}\right) > 0.05 \text{ or }
\]
\[
\text{if } p\text{ value of } \text{Binomial.test}(X = i, N = 2n, p = q_k, \text{right. tail}) \leq 0.05.
\]
\[
0, \text{if } p\text{ value of } \text{Binomial.test}\left(X = i, N = 2n, p = \frac{q_k}{\sqrt{Q}}, \text{left. tail}\right) \leq 0.05 \text{ and }
\]
\[
\text{if } p\text{ value of } \text{Binomial.test}(X = i, N = 2n, p = q_k, \text{right. tail}) > 0.05
\]

\(f_X(x = i)\) was computed using the \texttt{dbinom} function in R, and the binomial test p-value was computed with the \texttt{pbinom} function in R. The test power of the scenarios with \(n\) ranging from 50 to 1000, \(Q\) ranging from \(2 \times 10^{-4}\) to \(5 \times 10^{-3}\) under the AD mode and from \(1 \times 10^{-5}\) to \(1 \times 10^{-3}\) under the AR mode, and \(q_k\) ranging from \(1 \times 10^{-5}\) to \(5 \times 10^{-4}\) under the AD mode and from \(1 \times 10^{-4}\) to \(5 \times 10^{-3}\) under the AR mode were computed. The test specificity of the scenarios with \(n\) ranging from 50 to 1000, \(Q\) ranging from \(2 \times 10^{-4}\) to \(5 \times 10^{-3}\) under the AD mode and from \(1 \times 10^{-5}\) to \(1 \times 10^{-3}\) under the AR mode, and \(q_k\) ranging from \(5 \times 10^{-6}\) to \(1 \times 10^{-4}\) under the AD mode and from \(5 \times 10^{-5}\) to \(1 \times 10^{-3}\) under the AR mode were computed (Supplementary table S1, Figure 1).

**Simulation of sampling bias**

In reality, the sampling of patients might be not completely random (e.g. the case of inbreeding), resulting in a skewed distribution of the observed number of the variant in the patient cohort that will impact the performance of the statistics. To estimate how our test will be affected by deviation from random sampling, we simulated the scenarios of biased sampling and performed the test as without sampling bias. Specifically, assume \(r = \)The ratio of the sampled AF in patient cohort vs. the true AF in patient cohort

When computing test power, to simulate benign alleles, we set \(f_X(x = i)\) as the probability of observing \((x = i)\) count of \(a_k\) in a patient cohort with a sample size of \(2n\) (AR model) or \(n\) (AD model) given an occurrence rate of \(q_k \times r\) (AR model) or \(2q_k \times r\) (AD model) following a binomial distribution.
When computing test specificity, to simulate pathogenic alleles, we set $f_X (x = i)$ as the probability of observing $\left( x = i \right)$ count of $a_k$ in a patient cohort with a sample size of $2n$ (AR model) or $n$ (AD model) given an occurrence rate of $\frac{q_k}{\sqrt{Q}} \times r$ (AR model) or $\frac{q_k}{1-\sqrt{1-Q}} \times r$ (AD model) following a binomial distribution.

The test power and specificity were computed under the scenarios with $n$ ranging from 50 to 1000, $Q$ of 0.001 for the AD mode and of 0.0001 for the AR mode, $q_k$ of $5 \times 10^{-5}$ and $1 \times 10^{-4}$ for the AD mode and of $5 \times 10^{-4}$ and $1 \times 10^{-3}$ for the AR mode, and $r$ ranging from 10% to 500% (Supplementary table S2, Figure 2).

**Simulation of mis-specification of disease prevalence**

We evaluated the test performance for the scenarios of mis-specification of disease prevalence. We simulated the scenario with the accurate disease prevalence and performed the test with over- or under-estimated disease prevalence. We tested the scenarios with $n$ ranging from 50 to 1000, $Q$ of 0.001 under AD mode and of 0.0001 under AR mode, the ratio of estimated $Q$ vs the true $Q$ ranging from 10% to 20000%, $q_k$ of $5 \times 10^{-5}$ and $1 \times 10^{-4}$ under the AD mode and of $5 \times 10^{-4}$ and $1 \times 10^{-3}$ under the AR mode (Supplementary table S3, Supplementary Figure S1).

**Simulation of allele penetrance**

We evaluated the test power and specificity for the scenarios of alleles with various penetrance. We simulated the scenario of alleles with different penetrance values and performed the test considering alleles with complete penetrance.

According to the Bayesian model of allele penetrance:

$$P(D|A) = \text{Allele penetrance}$$

$$P(D) = \text{The disease prevalence in the general population}$$

$$P(A|D) = \text{Allele frequency in patients}$$
\[ P(A) = \text{Allele frequency in population} \]

Thus, for AD model:

\[
AF \text{ in the patient sample} = q_k \times \text{penetrance} \times \frac{1}{Q} \\
\approx \frac{q_k}{2(1-\sqrt{1-Q})} \times \text{penetrance}
\]

For autosomal recessive model:

\[
AF \text{ in the patient sample} = q_k \times \sqrt{Q} \times \frac{1}{Q} \\
= \frac{q_k}{\sqrt{Q}} \times \text{penetrance}
\]

Namely, to simulate the allele penetrance, it is basically to multiply the AF with the allele penetrance.

Assume \( j = \text{allele penetrance} \)

When computing test power, to simulate benign alleles, we set \( f_x(x = i) \) as the probability of observing \( (x = i) \) count of a benign allele \( a_k \) in a patient cohort with a sample size of \( 2n \) (AR model) or \( n \) (AD model) given an occurrence rate of \( q_k \) (AR model) or \( 2q_k \) (AD model) following a binomial distribution.

When computing test specificity, to simulate pathogenic alleles, we set \( f_x(x = i) \) as the probability of observing \( (x = i) \) count of a pathogenic allele \( a_k \) in a patient cohort with a sample size of \( 2n \) (AR model) or \( n \) (AD model) given an occurrence rate of \( \frac{q_k}{\sqrt{Q}} \times j \) (AR model) or \( \frac{q_k}{1-\sqrt{1-Q}} \times j \) (AD model) following a binomial distribution.

We tested scenarios with \( n \) ranging from 50 to 1000, \( Q \) of 0.001 under AD mode and of 0.0001 under AR mode, allele penetrance \( j \) ranging from 10% to 100%, \( q_k \) of \( 5 \times 10^{-5} \) and \( 1 \times 10^{-4} \) under the AD mode and of \( 5 \times 10^{-4} \) and \( 1 \times 10^{-3} \) under the AR mode (Supplementary table S4, Supplementary Figure S2).
Simulation of a scenario of a disease attributed to alleles with heterogeneous penetrance

We estimated the test power and specificity under the scenarios of a disease attributed to four types of alleles with heterogeneous penetrance. Specifically, we assume

\[ q_1 = \text{the population AF of type 1 allele} \]
\[ q_2 = \text{the population AF of type 2 allele} \]
\[ q_3 = \text{the population AF of type 3 allele} \]
\[ q_4 = \text{the population AF of type 4 allele} \]
\[ j_1 = \text{the penetrance of type 1 allele} \]
\[ j_2 = \text{the penetrance of type 2 allele} \]
\[ j_3 = \text{the penetrance of type 3 allele} \]
\[ j_4 = \text{the penetrance of type 4 allele} \]

Thus, for AD model:

\[
\frac{q_1 \times j_1}{1-\sqrt{1-Q}} + \frac{q_2 \times j_2}{1-\sqrt{1-Q}} + \frac{q_3 \times j_3}{1-\sqrt{1-Q}} + \frac{q_4 \times j_4}{1-\sqrt{1-Q}} = 1
\]

This equation can have multiple solutions. For simplification, we chose two of the possible solutions.

The first solution is for the scenario where the allele population AF is negative correlated with penetrance. Let

\[ q_1 = q_h \times 0.5/j_1 \]
\[ q_2 = q_h/j_2 \]
\[ q_3 = q_h/j_3 \]
\[ q_4 = q_h/j_4 \]

Namely,

\[
\frac{q_h \times 0.5/j_1}{1-\sqrt{1-Q}} + \frac{q_h/j_2 \times j_2}{1-\sqrt{1-Q}} + \frac{q_h/j_3 \times j_3}{1-\sqrt{1-Q}} + \frac{q_h/j_4 \times j_4}{1-\sqrt{1-Q}} = 1
\]

Thus,

\[ q_h = \frac{(1 - \sqrt{1-Q})}{3.5} \]
\[ q_{k1} = \frac{0.5 \times \sqrt{1 - Q}}{3.5} , \quad q_{k2} = \frac{1 - \sqrt{1 - Q}}{3.5} , \quad q_{k3} = \frac{1 - \sqrt{1 - Q}}{3.5} , \quad q_{k4} = \frac{1 - \sqrt{1 - Q}}{3.5} \]

We set \( Q \) at 0.001, \( j_1 \) at 0.2, \( j_2 \) at 0.5, \( j_3 \) at 0.8, \( j_4 \) at 1, thus,

\[ q_{k1} = \frac{0.5 \times \sqrt{1 - 0.001}}{0.2} , \quad q_{k2} = \frac{1 - \sqrt{1 - 0.001}}{0.5} , \quad q_{k3} = \frac{1 - \sqrt{1 - 0.001}}{0.8} , \quad q_{k4} = \frac{1 - \sqrt{1 - 0.001}}{1} \]

We assume each of the four allele types include five alleles, thus the population AF of each allele will be

\[ \frac{q_{k1}}{5} , \frac{q_{k2}}{5} , \frac{q_{k3}}{5} , and \frac{q_{k4}}{5} \]

The second solution is for the scenario where the population AF of alleles with heterogenous penetrance are similar. Let

\[ q_{k1} = q_{k2} = q_{k3} = q_{k4} = q_h \]

Namely,\[ \frac{q_h \times j_1}{1 - \sqrt{1 - Q}} + \frac{q_h \times j_2}{1 - \sqrt{1 - Q}} + \frac{q_h \times j_3}{1 - \sqrt{1 - Q}} + \frac{q_h \times j_4}{1 - \sqrt{1 - Q}} = 1 \]

We set \( Q \) at 0.001, \( j_1 \) at 0.2, \( j_2 \) at 0.5, \( j_3 \) at 0.8, \( j_4 \) at 1, thus,

\[ q_{k1} = q_{k2} = q_{k3} = q_{k4} = q_h = \frac{1 - \sqrt{1 - 0.001}}{(0.2 + 0.5 + 0.8 + 1)} \]

We assume each of the four allele types include five alleles, thus the population AF of each allele will be

\[ \frac{q_{k1}}{5} , \frac{q_{k2}}{5} , \frac{q_{k3}}{5} , and \frac{q_{k4}}{5} \]

Similarly, for AR model:

\[ \frac{q_{k1} \times j_1}{\sqrt{Q}} + \frac{q_{k2} \times j_2}{\sqrt{Q}} + \frac{q_{k3} \times j_3}{\sqrt{Q}} + \frac{q_{k4} \times j_4}{\sqrt{Q}} = 1 \]

This equation can have multiple solutions. For simplification, we chose two of the possible solutions.
The first solution is for the scenario where the allele population AF is negative correlated with penetrance. Let

\[ q_{k1} = q \times 0.5/j_1 \]
\[ q_{k2} = q/j_2 \]
\[ q_{k3} = q/j_3 \]
\[ q_{k4} = q/j_4 \]

Namely,

\[ \frac{0.5 \times q \times j_1}{\sqrt{Q}} + \frac{q \times j_2}{\sqrt{Q}} + \frac{q \times j_3}{\sqrt{Q}} + \frac{q \times j_4}{\sqrt{Q}} = 1 \]

Thus,

\[ q = \sqrt{Q}/3.5 \]
\[ q_{k1} = \frac{0.5 \times \sqrt{Q}}{j_1}, \quad q_{k2} = \frac{\sqrt{Q}}{j_2}, \quad q_{k3} = \frac{\sqrt{Q}}{j_3}, \quad q_{k4} = \frac{\sqrt{Q}}{j_4} \]

We set \( Q \) at 0.0001, \( i_1 \) at 0.2, \( i_2 \) at 0.5, \( i_3 \) at 0.8, \( i_4 \) at 1, thus,

\[ q_{k1} = \frac{0.5 \times 0.0001}{0.2}, \quad q_{k2} = \frac{\sqrt{0.0001}}{0.5}, \quad q_{k3} = \frac{\sqrt{0.0001}}{0.8}, \quad q_{k4} = \frac{\sqrt{0.0001}}{1}. \]

We assume each of the four allele types include five alleles, thus the population AF of each allele will be

\[ \frac{q_{k1}}{5}, \frac{q_{k2}}{5}, \frac{q_{k3}}{5}, and \frac{q_{k4}}{5} \]

The second solution is for the scenario where the population AF of alleles with heterogenous penetrance are similar. Let

\[ q_{k1} = q_{k2} = q_{k3} = q_{k4} = q_h \]

Namely,

\[ \frac{q_h \times j_1}{\sqrt{Q}} + \frac{q_h \times j_2}{\sqrt{Q}} + \frac{q_h \times j_3}{\sqrt{Q}} + \frac{q_h \times j_4}{\sqrt{Q}} = 1 \]

We set \( Q \) at 0.0001, \( i_1 \) at 0.2, \( i_2 \) at 0.5, \( i_3 \) at 0.8, \( i_4 \) at 1, thus,

\[ q_{k1} = q_{k2} = q_{k3} = q_{k4} = q_h = \frac{\sqrt{0.0001}}{(0.2 + 0.5 + 0.8 + 1)} \]

We assume each of the four allele types include five alleles, thus the population AF of each allele will be
\[
\frac{q_{k1}}{5}, \frac{q_{k2}}{5}, \frac{q_{k3}}{5}, \text{and } \frac{q_{k4}}{5}
\]

Based on the above values of \(Q, j_1, j_2, j_3, j_4, q_{k1}/5, q_{k2}/5, q_{k3}/5, q_{k4}/5\), using the aforementioned formula derived for allele with various penetrance, we computed the test power and specificity. We tested scenarios with \(n\) ranging from 50 to 1000 under AD and AR mode (Supplementary table S4, Supplementary Figure S3).

**The collection and preprocessing of real data**

157 Stargardt (STGD) patients who are mainly attributed to ABCA4 gene and 107 Retinitis pigmentosa (RP) patients who are mainly attributed to candidate pathogenic variants in USH2A gene were collected upon variant screening for known inherited retinal disease genes using a customized capture panel\(^1\). The STGD and RP cohorts are mainly composed of patients of European decent. In addition, 96 East-Asian patients with Familial Exudative Vitreoretinopathy (FEVR) which are mainly caused by heterozygous LRP5 variants were collected through literature search\(^2-10\).

To screen the previously reported pathogenic variants in ABCA4, USH2A and LRP5, variants recorded in the HGMD database were extracted. For each HGMD variant, the population AF \((q_i)\) of the variant, was obtained from the gnomAD database (v.2.0.1) (downloaded from [http://gnomad-old.broadinstitute.org/downloads](http://gnomad-old.broadinstitute.org/downloads)). To match with the ethnicity of the collected patient cohorts, the Non-Finnish European AF was applied for the variants in ABCA4 and USH2A in the test, while the East-Asian AF was applied for the variants in LRP5 in the test. The corresponding disease prevalence \((Q)\) was found from literature\(^2,11-14\). The patient cohort size \((n)\) and the observed allele count \((x)\) in the patient cohort were computed according to the above collected STGD cohort \((n = 157)\), RP cohort \((n = 107)\) and FEVR cohort \((n = 96)\).

In our STGD cohort, 157 of 317 patients are likely to be solved by ABCA4 variants (i.e. carrying two pathogenic variants of ABCA4). Thus, we estimated that ABCA4 biallelic variants could attribute to about half of STGD patients. Given the disease prevalence of
STGD is about 1/10000\textsuperscript{11,12,15-17}, thus, the frequency of STGD patients due to \textit{ABCA4} bi-allelic variants could be estimated at 50\% \times \dfrac{1}{10000} (Q = \dfrac{1}{20000}). For each \textit{ABCA4} variants in HGMD, we determined whether the allele is likely to be benign by applying two binomial tests, \textit{Binom.test}\((X = x, N = 2 \times 157, p = \dfrac{q_i}{\sqrt{20000}}, \text{alternative} = \text{"less"})\) and \textit{Binom.test}\((X = x, N = 2 \times 157, p = q_i, \text{alternative} = \text{"greater"})\).

The disease frequency/prevalence of RP was estimated as 1 in 4000 individuals (https://ghr.nlm.nih.gov/condition/retinitis-pigmentosa#statistics). About 10\%-15\% of RP patients were estimated to be caused by the recessive variants in \textit{USH2A}\textsuperscript{12,13}. Thus, the frequency of RP patients due to variants in \textit{USH2A} was set at 11\% \times \dfrac{1}{4000} (Q = \dfrac{11}{400000}). For each \textit{USH2A} variants in HGMD, we determined whether the allele is likely to be benign by applying two binomial tests, binomial test \textit{Binom.test}\((X = x, N = 2 \times 107, p = \dfrac{q_i}{\sqrt{11 \times 100000}}, \text{alternative} = \text{"less"})\) and \textit{Binom.test}\((X = x, N = 2 \times 107, p = q_i, \text{alternative} = \text{"greater"})\).

Currently there is no systematically estimation of FEVR prevalence. The frequency of FEVR in screening the healthy full-term newborns is about 0.4\%\textsuperscript{14}. And 12\%-25\% of FEVR is estimated to attributed to \textit{LRP5}\textsuperscript{2,10}. \textit{LRP5} is mostly associated with dominant FEVR and occasionally with recessive FEVR\textsuperscript{2}. Therefore, in the test model, the frequency of autosomal dominant FEVR (adFEVR) patients due to heterozygous variant in \textit{LRP5} was set at 25\% \times 4/1000 (Q = \dfrac{1}{1000}). For each \textit{LRP5} variants in HGMD, we estimated whether the allele is likely to be benign by applying two binomial tests, \textit{Binom.test}\((X = x, N = 96, p = \dfrac{q_i}{\sqrt{1 - \dfrac{1}{1000}}}, \text{alternative} = \text{"less"})\) and \textit{Binom.test}\((X = x, N = 96, p = q_i \times 2, \text{alternative} = \text{"greater"})\).

The intermediate steps were implemented with R and PERL scripts.

\textbf{Comparison with other variant prediction scores}
To evaluate our test result using other variant prediction scores, we collected the CADD score\(^{18}\) (v1.4 https://cadd.gs.washington.edu/download), REVEL score\(^{19}\) (https://sites.google.com/site/revelgenomics/downloads), phastconservation score (http://hgdownload.cse.ucsc.edu/goldenpath/hg19/phyloP100way/hg19.100way.phyloP100way/), Clinvar assignment (ftp://ftp.ncbi.nlm.nih.gov/pub/clinvar/tab_delimited/variant_summary.txt.gz) for the reported \textit{ABCA4}, \textit{USH2A}, and \textit{LRP5} variants in HGMD.

\textbf{Luciferase assay}

To conduct functional validation on our test results, we performed a multiplex luciferase assay on five \textit{LRP5} variants that were predicted to be likely benign along with wild type and positive and negative controls\(^{18}\). The investigator who performed the experiment was not aware of the pathogenicity of the five \textit{LRP5} variants. HEK293T and L-cells were used during \textit{in vitro} luciferase assay of \textit{LRP5} function. HEK293T and L-cells were grown in complete medium, consisting of DMEM High Glucose (Sigma D5796) supplemented with 10\% Fetal Bovine Serum (Gibco 26140-079). L-cells were kindly provided by the lab of Dr. David Moore at Baylor College of Medicine. \textit{WNT3A} and control conditioned medium were made by culturing L-cells overexpressing \textit{WNT3A} and wildtype L-cells. L-cells were plated in 10 cm dishes (Cellstar 664160) at 10\% confluency to grow in complete medium for 4 days. The first batch of medium was collected and saved, then fresh medium was added to the same culture. After an additional 3 days, the second batch of medium was collected and combined 1:1 with the first batch of medium to form the final conditioned medium (either control or \textit{WNT3A} conditioned medium). Twenty-four hours prior to transfection of \textit{LRP5} variant or control luciferase reporter construct, HEK293T cells were plated in 96-well plates (Corning Costar 3595) at 60\% confluency. Cells were moved from complete medium to either
control or WNT3A conditioned medium then transfected with 100 ng LRP5 variant or control constructs using TransIT-293 transfection reagent (Mirus) and cultured for 24 hours. Media was removed and passive lysis buffer (Promega E194A) was applied for cell lysate extraction. Cell lysates were measured for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega E1980) followed by the recording of light emission using the CLARIOstar microplate reader (BMG LABTECH). Experiments were performed in triplicates (n ≥ 3). In addition, details related to the construction of the LRP5 constructs and reporter assay will be published elsewhere.

Supplementary Results:

Simulation analysis of misspecification of disease prevalence

If the disease prevalence is underestimated, the test power to detect benign alleles and specificity to detect pathogenic alleles will not be significantly affected (Supplementary Figure S1 and Table S3). For example, for an AD disease with a prevalence of 1/1000 and variants with a population AF of 5×10⁻⁵ or 1×10⁻⁴, when the disease prevalence is underestimated to various extent, the test power to detect benign alleles and the test specificity to detect pathogenic alleles are close to 100% with a sample size as low as 50. The similar trend was observed under the AR model (Supplementary Figure S1, Table S3).

If the disease prevalence is overestimated, the test power to detect benign alleles will decrease but will rapidly recover as the sample size increases, while test specificity to detect pathogenic alleles will not be significantly affected (Supplementary Figure S1 and Table S3). For example, for an AD disease with a disease prevalence of 1/1000, when the disease prevalence is overestimated as 10 folds (1000%) of the true disease prevalence, the test power to detect benign alleles with a population AF of 5×10⁻⁵ will increase from 0 to about 97% when the sample size increases from 250 to 300. Whereas, when the disease prevalence is overestimated to various extent, the test specificity is close to 100%. The similar trend was observed under the AR model as well (Supplementary Figure S1, Table S3).
Simulation analysis of allele penetrance

We performed the simulation for the scenarios of alleles with various penetrance. Based on the simulation, the test power to detect benign alleles is not significantly affected, and test specificity to detect pathogenic alleles does decrease for alleles with low penetrance using small patient cohort sizes but will rapidly improve as the sample size increases (Supplementary Figure S2 and Table S4). For example, under the AD model, for a disease with a prevalence of 1 in 1000 and variants with a population AF of $5 \times 10^{-5}$, the test power is close to 100% for alleles with various extent of penetrance using a sample size as low as 50. Whereas, when allele penetrance is only 20%, the test specificity to detect pathogenic alleles with a population AF of $5 \times 10^{-5}$ will increase from 64% to about 91% when the sample size increases from 50 to 120. The similar trend was observed under the AR model (Supplementary Figure S2, Table S4).

Furthermore, we conducted the simulation for the scenario of a disease attributed to multiple pathogenic alleles with heterogenous penetrance. Under this scenario, the test power to detect benign alleles is not significantly affected, and test specificity to detect pathogenic alleles does decrease using small sample sizes for alleles with low penetrance but can be rapidly improved as the sample size increases (Supplementary Figure S3 and Table S4). For example, under the AD model, for a disease with a prevalence of 1 in 1000 and variants with a population AF of $5.72 \times 10^{-5}$, the test power is close to 100% for alleles with an allele penetrance of 50% using a sample size of 50. For variants with rarer AF, larger sample size can allow the test to achieve high power. On the other hand, when allele penetrance is only 20%, the test specificity to detect pathogenic alleles with a population AF of $4 \times 10^{-5}$ will increase from 55% to about 96% when the sample size increases from 50 to 200. Overall, the test shows excellent power and specificity for alleles with penetrance $\geq 50\%$ (Supplementary Figure S3, Table S4).

Estimation of the thresholds of population allele frequency for filtering benign variants
We performed simulation to determine the thresholds of population allele frequency for filtering benign variants for diseases with a variety of disease prevalence under the AD/AR model, respectively. As shown in Supplementary Table S5, using a patient cohort with a size of 50, under the AR model, applying the AF thresholds ranging from 0.01% to 0.15% can achieve above 95% test power and 95% test specificity to filter benign alleles for the diseases with a prevalence ranging from 1/100000 to 1/2000. Under the AD model, applying the AF thresholds ranging from 0.0003% to 0.015% can achieve above 98% test power and 95% test specificity to filter benign alleles for the diseases with a prevalence ranging from 1/10000 to 1/200. If using a patient cohort with larger sample size, e.g. a size of 100, the more stringent AF thresholds can be achieved (Supplementary Table S5).

Our test allows the filtering of alleles that are at least 10 times less frequent in population than applying thresholds based on disease prevalence alone (namely, using $\sqrt{Q}$ for AR diseases and for $Q/2$ for AD diseases). As a result, a significant amount of rare variants passing current frequency filter (based on disease prevalence alone) can be excluded by our method, by applying our AF thresholds to 10 WES (Caucasians) of our inherited retinal disease (Mendelian diseases) patient cohort, based on the allele frequency in non-Finnish European population in the gnomAD database (Supplementary Table S5).

**Other variant prediction scores support our test results**

Many computational prediction tools have been developed to distinguish deleterious from benign variants. Although none of the tools is perfect, many of them achieved adequate performance for a large proportion of variants. Therefore, it is reasonable to evaluate our predicted results by comparing with the results obtained from these computational tools. We have selected three commonly used tools, including CADD, REVEL and phastcon, for the evaluation.

CADD score is a popular score for predicting the deleteriousness of genetic variants by integrating multiple annotations. The higher CADD score indicates the associated
variant is more likely to be deleterious. For example, a scaled CADD score of 20 suggests that the associated variant has the deleteriousness among the top 1% of all single nucleotide variants (SNVs) in the human genome, and a scaled CADD score of 30 indicates among the top 0.1% of all SNVs. The comparison of the CADD scores show that the scaled CADD scores of the variants identified as benign by our test are significantly lower than those of the other HGMD variants in the same gene, suggesting the putative benign variants are less deleterious and supporting our test result. Specifically, as shown in Figure 4A, the ABCA4 variants that are identified as benign have a mean scaled CADD score of 18.28 compared to 28.48 for the rest of ABCA4 variants in HGMD (Wilcoxon test, one tailed p-value = 4.31e-12). Similarly, significantly lower CADD score is observed for USH2A and LRP5 when comparing benign variants predicted by our method to the rest of the HGMD variants in the same gene (Figure 4A and Supplementary table S6, S8, S10, S12).

Consistent with CADD score, the benign variants identified by our method also have lower REVEL and phastcon_100way scores than the other HGMD variants in the same gene for all the three genes (Figure 4B and 4C, Supplementary table S6, S8, S10, S12). In addition, it is interesting to note that the difference in conservation between the likely benign and pathogenic variants is relatively small. For example, for USH2A, the identified likely benign variants have a mean phastcon score of 0.80, while the other HGMD variants have a mean phastcon score of 0.84 (Supplementary table S9). Furthermore, it is notable that some of the likely benign variants has a high CADD and REVEL score. For example, ABCA4:c.G1610A:p.R537H is predicted to be benign by our test, which is also supported by its maximum subpopulation AF of 1.31×10^{-2}, the presence in homozygous in two individuals in gnomAD, and the classification as likely benign by ClinVar; but it has a scaled CADD score of 25.9 and a REVEL score of 0.715 (Supplementary table S6). Additionally, REVEL is not available for noncoding, stop-gain and frameshift variants. Therefore, these results suggest that the current computational methods are the right trend but not perfect.
In addition, the ClinVar assessment largely supports our test results as well. For the 37 variants predicted to be benign by our test in *ABCA4*, only two were classified as pathogenic/likely pathogenic. Similarly, no variant in *USH2A* and only one variant in *LRP5* is classified as likely pathogenic in ClinVar (Figure 4D).

**Functional assay of predicted benign variants in LRP5**

To further evaluate our results, we performed a functional assay on five *LRP5* variants that were originally reported as pathogenic but are identified as likely benign by our test. Three of the five variants show no effect on LRP5 function, suggesting they are likely benign. Additionally, one variant shows mild negative effect on LRP5 function while another variant shows deleterious effect on LRP5 function (Figure 5, supplementary table S13).

Specifically, five variants, i.e. p. A422V, p. R1219H, p.R1342Q, p.H1383P, p.T1506M, were tested to determine whether they affect the ability of LRP5 to activate WNT signaling. Multiple controls, including one positive control, i.e. p. Q368X (a stop-gained variant), one negative control, i.e. p. D666N (a variant with maximum subpopulation frequency = 0.37% and have one homozygote in the gnomAD database), and the wildtype allele, are tested in parallel. *LRP5* cDNA carrying an individual variant is transfected in HEK293T cells and the resulting WNT signaling activity without or with the treatment of WNT3A is measured using a luciferase assay. After normalizing the signaling activity against control signaling, the positive control *LRP5*.Q368X has significantly reduced WNT signaling activity with or without WNT3A treatment (two tailed T test, p-value < 1e-5), while the negative control *LRP5*.D666N is higher than the baseline wild type cDNA without WNT3A (two tailed T test, p=2.6e-4) and similar to the wild type upon WNT3A treatment (two tailed T test, p-value=0.7132), suggesting the luciferase reporter system is working appropriately. Three of the variants, *LRP5*.R1219H (two tailed T test, p-value < 0.005), *LRP5*.R1342Q (two tailed T test, p-value > 0.05), *LRP5*.H1383P (two tailed T test, p-value < 0.002), have similar or higher WNT signaling activity than the wild type control without or with WNT3A treatment (Figure 5), indicating these three variants are likely benign. In contrast, *LRP5*.A422V
show similar signaling activity to \textit{LRP5.WT} without WNT3A treatment (two tailed T test, p-value = 0.2733), but its activity is reduced by about 50% with WNT3A treatment (two tailed T test, p-value = 4.95e-5) (Figure 5), suggesting that \textit{LRP5.A422V} is likely to be a hypomorphic allele. This is consistent with the original report that this variant is \textit{in-cis} with \textit{LRP5.R348W} in multiple members of a family\textsuperscript{19} and therefore might not be severe enough to lead to a phenotype alone. Finally, \textit{LRP5.T1506M} shows lower signaling activity than \textit{LRP5.WT} without (two tailed T test, p-value = 1.01e-6) or with WNT3A treatment (two tailed T test, p-value = 1.31e-7), suggesting p.T1506M is likely to be a pathogenic allele (Figure 8, supplementary table S13).
Supplemental Figures

Supplementary Figure S1. The simulation of misspecification of disease prevalence
Supplementary Figure S2. The simulation of alleles with various penetrance
**Supplementary Figure S3.** The simulation of diseases attributed to alleles with heterogenous allele penetrance and different or similar AF.
**Supplementary Tables (.xls format)**

Supplementary table S1. The simulation data without sampling bias under a variety of parameter settings.

Supplementary table S2. The simulation data for sampling bias.

Supplementary table S3. The simulation data of misspecification of disease prevalence.

Supplementary table S4. The simulation data of the impact of allele penetrance.

Supplementary table S5. The simulation of population AF thresholds for filtering benign alleles.

Supplementary Table S6. *ABCA4* variants.
This table lists the information of 945 *ABCA4* variants in HGMD, including genomic positions, alteration, the allele count and allele frequency in our STGD patient cohort, and the maximum subpopulation allele count/allele frequency/allele number, the NFE population allele count/allele frequency/allele number, and the number of homozygotes in gnomAD, our binomial test p values, phastcon score, raw and scaled CADD score, ClinVar assignment, and REVEL score. The predicted likely benign variants were highlighted in red.
Supplementary Table S7. The summary of the review of the original papers associated with the 26 ABCA4 variants that are identified as likely benign variants by our test.

Supplementary Table S8. USH2A variants.
This table lists the information of 748 USH2A variants in HGMD. The variants that were predicted likely benign were highlighted in red.

Supplementary Table S9. The summary of the review of the original papers associated with the 18 USH2A variants that are identified as likely benign variants by our test.

Supplementary Table S10. LRP5 variants.
This table lists the information of 150 LRP5 variants in HGMD. The variants that were predicted likely benign were highlighted in red.

Supplementary Table S11. The summary of the review of the original papers associated with the 16 LRP5 variants that are identified as likely benign variants by our test.

Supplementary Table S12. The comparison of the CADD, REVEL and phastcon scores between putative benign variants and the rest variants in HGMD for ABCA4, USH2A, and LRP5.

Supplementary Table S13. The Luciferase reporter assay data
Reference:

1. Xu M, Eblimit A, Wang J, et al. ADIPOR1 Is Mutated in Syndromic Retinitis Pigmentosa. *Hum Mutat.* 2016;37(3):246-249.

2. Li JK, Li Y, Zhang X, et al. Spectrum of Variants in 389 Chinese Probands With Familial Exudative Vitreoretinopathy. *Invest Ophthalmol Vis Sci.* 2018;59(13):5368-5381.

3. Huang XY, Zhuang H, Wu JH, et al. Targeted next-generation sequencing analysis identifies novel mutations in families with severe familial exudative vitreoretinopathy. *Mol Vis.* 2017;23:605-613.

4. Rao FQ, Cai XB, Cheng FF, et al. Mutations in LRP5,FZD4, TSPAN12, NDP, ZNF408, or KIF11 Genes Account for 38.7% of Chinese Patients With Familial Exudative Vitreoretinopathy. *Invest Ophthalmol Vis Sci.* 2017;58(5):2623-2629.

5. Fei P, Zhang Q, Huang L, et al. Identification of two novel LRP5 mutations in families with familial exudative vitreoretinopathy. *Mol Vis.* 2014;20:395-409.

6. Yang H, Li S, Xiao X, Wang P, Guo X, Zhang Q. Identification of FZD4 and LRP5 mutations in 11 of 49 families with familial exudative vitreoretinopathy. *Mol Vis.* 2012;18:2438-2446.

7. Liu YQ, Zhu X, Li SJ, et al. Identification of LRP5 mutations in families with familial exudative vitreoretinopathy. *Yi Chuan.* 2017;39(3):241-249.

8. Chen C, Wang Z, Sun L, et al. Next-Generation Sequencing in the Familial Exudative Vitreoretinopathy-Associated Rhegmatogenous Retinal Detachment. *Invest Ophthalmol Vis Sci.* 2019;60(7):2659-2666.

9. Tang M, Sun L, Hu A, et al. Mutation Spectrum of the LRP5, NDP, and TSPAN12 Genes in Chinese Patients With Familial Exudative Vitreoretinopathy. *Invest Ophthalmol Vis Sci.* 2017;58(13):5949-5957.

10. Seo SH, Yu YS, Park SW, et al. Molecular Characterization of FZD4, LRP5, and TSPAN12 in Familial Exudative Vitreoretinopathy. *Invest Ophthalmol Vis Sci.* 2015;56(9):5143-5151.

11. Blacharski P. *Fundus flavimaculatus.* In: Newsome DA, (ed). *Retinal dystrophies and degenerations.* New York: Raven Press; 1988.

12. Huang L, Mao Y, Yang J, Li Y, Li Y, Yang Z. Mutation screening of the USH2A gene in retinitis pigmentosa and USHER patients in a Han Chinese population. *Eye (Lond).* 2018;32(10):1608-1614.

13. Sandberg MA, Rosner B, Weigel-DiFranco C, McGee TL, Dryja TP, Berson EL. Disease course in patients with autosomal recessive retinitis pigmentosa due to the USH2A gene. *Invest Ophthalmol Vis Sci.* 2008;49(12):5532-5539.

14. Li LH, Li N, Zhao JY, et al. Findings of perinatal ocular examination performed on 3573, healthy full-term newborns. *Br J Ophthalmol.* 2013;97(5):588-591.

15. Fujinami K, Lois N, Davidson AE, et al. A longitudinal study of stargardt disease: clinical and electrophysiologic assessment, progression, and genotype correlations. *Am J Ophthalmol.* 2013;155(6):1075-1088 e1013.

16. Michaelides M, Hunt DM, Moore AT. The genetics of inherited macular dystrophies. *J Med Genet.* 2003;40(9):641-650.

17. Zernant J, Xie YA, Ayuso C, et al. Analysis of the ABCA4 genomic locus in Stargardt disease. *Hum Mol Genet.* 2014;23(25):6797-6806.
18. Sarrion-Perdigones A, Chang L, Gonzalez Y, Gallego-Flores T, Young DW, Venken KJT. Examining multiple cellular pathways at once using multiplex hextuple luciferase assaying. *Nat Commun.* 2019;10(1):5710.

19. Salvo J, Lyubasyuk V, Xu M, et al. Next-generation sequencing and novel variant determination in a cohort of 92 familial exudative vitreoretinopathy patients. *Invest Ophthalmol Vis Sci.* 2015;56(3):1937-1946.