Supplementary Data

Association Between Monoallelic TSHR Mutations and Congenital Hypothyroidism: A Statistical Approach

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Supplementary Methods

Functional analyses

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Sigma, St. Louis, MO, USA) supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin and 10% fetal bovine serum.

The wildtype-TSHR expression vector has been described (1). Expression vectors encoding hemagglutinin (HA)-tagged DUOX2 (HA-DUOX2) and myc-tagged DUOXA2 (DUOXA2-myc) were provided by H. Grasberger (2). Three TSHR variants (R519H, L669H and A705Dfs*24) and three DUOX2 variants (E327*, K530* and V779M) were introduced by site-directed mutagenesis (QuickChange XL II Site-Direct Mutagenesis kit; Agilent, Santa Clara, CA, USA).

For assessment of TSHR variants (R519H, L669H and A705Dfs*24), we seeded HEK293 cells into a 96-well plate at 70-80% confluence, and transfected the cells using Lipofectamine 2000 (Invitrogen, Waltham, MA, USA) with 10 ng of each TSHR expressing construct (wildtype or mutant) along with 50 ng of the reporter vector encoding the CRE-luciferase construct (pGL4.29, Promega, Madison, WI, USA). Forty-eight hours after transfection, the cells were incubated with or without 100 U/L bovine TSH (Sigma) in DMEM for 3 hours at 37°C. The luciferase activity was measured using the ONE-Glo Luciferase Assay System (Promega).

For assessment of DUOX2 variants (E327*, K530* and V779M), H2O2-producing capacities were studied in the presence of DUOXA2 with use of Amplex Red kit (Life Technologies, Carlsbad, CA, USA). HEK293 cells were seeded in a 24-well plate and were transfected with DNA (HA-DUOX2 300 ng plus DUOXA2-myc 100 ng) at 70-80% confluence. Forty-eight hours after transfection, cells were harvested, were washed with Phosphate buffered saline (PBS) and were resuspended in 100 μl of Earle’s balanced salt solution (Sigma). We measured extracellular H2O2 production on cells resuspended in the solution with the 1 μM ionomycin (Sigma) and the Amplex Red reagent (Life Technologies).

Statistical analysis

The frequency of permanent CH and transient CH due to biallelic DUOX2 mutations has been reported to be 1:44,000 and 1:29,600, respectively (3, 4). We estimated the the frequency of biallelic and monoallelic DUOX2 mutation. DUOX2 p.H678R is a functional SNP (rs57659670, allele frequency of Japanese 0.067) (4). We calculated the frequency of double heterozygotes in situation that DUOX2 p.H678R is regarded as the mutation.

To estimate the probability of newborn screening (NBS) positivity given mutation carriers, we used a Bayes' theorem as follows (5, 6).

We defined the parameters as

\[ P(X) \] probability of \( X \)
\[ P(Y) \] probability of \( Y \)
\[ P(X|Y) \] conditional probability of \( X \) given \( Y \)

C: Positive NBS result for CH (NBS positivity)
N: Negative NBS result for CH
\( M_{TSHR} \): carrying monoallelic \( TSHR \) mutation
M<sub>TSR/DUOX2</sub>: carrying double heterozygous mutations in TSHR and DUOX2

By the Bayesian method,
\[
P(X|Y) = \frac{P(Y|X)P(X)}{P(Y)}
\]
\[
P(C|\mathcal{M}) = \frac{P(M|C)P(C)}{P(M)} = \frac{P(M|C)P(C)}{P(M|C)P(C)+P(M|\neg C)P(\neg C)}
\]

**Supplementary Results**

*The frequency of double heterozygotes (monoallelic mutations in TSHR and DUOX2) in the Japanese general population.*

The frequency of biallelic DUOX2 mutations was estimated to be 1:17,700 (1/44,000 + 1/29,600) and monoallelic DUOX2 mutations in the general population was estimated to be 1:67 based on the frequency of biallelic DUOX2 mutations and Hardy-Weinberg equation. The frequency of double heterozygotes was estimated to be 0.0087% (1:11,524 [1/172×1/67]).

\[
P(C) : \text{probability of positive NBS result for CH} = \frac{1}{2975}
\]
\[
P(N) : \text{probability of negative NBS result for CH} = \frac{2974}{2975}
\]
\[
P(M_{TSR}|C) : \text{probability of monoallelic TSHR mutation carriers conditional on positive NBS} = \frac{26}{395} = 0.066
\]
\[
P(M_{TSR}|N) : \text{probability of monoallelic TSHR mutation carriers conditional on negative NBS} = \frac{1}{172} = 0.0058
\]
\[
P(M_{DUOX2|C}) : \text{probability of double heterozygotes conditional on positive NBS} = \frac{4}{395} = 0.010
\]
\[
P(M_{DUOX2|\neg C}) : \text{probability of double heterozygotes conditional on negative NBS} = \frac{1}{11524} = 0.000087
\]

The posterior conditional probability of NBS positivity given the monoallelic TSHR mutation carriers was

\[
P(C|M_{TSR}) = \frac{P(M_{TSR}|C)P(C)}{P(M_{TSR}|C)P(C)+P(M_{TSR}|N)P(N)} = \frac{26}{395} \times \frac{1}{2975} = -0.0038 \text{ (0.38%)}
\]

The posterior conditional probability of NBS positivity given the double heterozygotes was

\[
P(C|M_{TSR/DUOX2}) = \frac{P(M_{TSR/DUOX2}|C)P(C)}{P(M_{TSR/DUOX2}|C)P(C)+P(M_{TSR/DUOX2}|\neg C)P(\neg C)} = \frac{4}{395} \times \frac{1}{2975} = -0.038 \text{ (3.8%)}
\]

**Odds ratio calculation and posterior probability with taking DUOX2 p. H678R into account**

The frequency of double heterozygotes contained DUOX2 p.H678R as a mutation in general population
The frequency of double heterozygotes including DUOX2 p.H678R as a mutation was 1.5% (6 in 395) (95% CI, 0.56-3.3%), which was significantly higher than that in the Japanese general population (P<0.05, Z test for H₀: true frequency=0.087%). The OR for NBS positivity associated with double heterozygotes was 18.

We estimated the posterior probability of NBS positivity given the double heterozygotes. We defined parameters as

\[
P(M_{\text{TSR/DUOX2}}|C) : \text{probability of double heterozygotes conditional on positive NBS} = \frac{6}{395} = 0.015
\]

\[
P(M_{\text{TSR/DUOX2}}|N) : \text{probability of double heterozygotes conditional on negative NBS} = 0.000865
\]

The posterior conditional probability of NBS positivity given the double heterozygotes was

\[
P(C|M_{\text{TSR/DUOX2}}) = \frac{P(M_{\text{TSR/DUOX2}}|C)P(C)}{P(M_{\text{TSR/DUOX2}}|C)P(C)+P(M_{\text{TSR/DUOX2}}|N)P(N)} = \frac{\frac{6}{395} \times \frac{1}{172}}{\frac{6}{395} \times \frac{1}{172} + 0.000865 \times \frac{2974}{2975}} = 0.0056 (0.59%)
\]

References


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