Consecutive Mutational Events in a *TSHR* Allele of Arab Families with Resistance to Thyroid Stimulating Hormone

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Background: Our laboratory identified six distinct inactivating *TSHR* gene mutations in Arab tribes living in Israel. We recently reported three nucleotide substitutions in exon 3 producing p.[L89L;Q90P] and one in exon 9 of the same allele producing p.P264S in Family A. Family B, reported herein, harbors the identical mutation in exon 3 only. We set to determine whether the mutations have common ancestral origin.

Methods: Coding regions of the *TSHR* were sequenced and flanking microsatellite markers spanning 5.3 cM were used for haplotyping.

Results: Two siblings of Family B were compound heterozygous for *TSHR* gene mutations. The paternal allele contained the exon 3 mutation and the maternal allele harbored a mutation in exon 10 (p.L653V). We investigated the possibility of a founder effect with subsequent mutational events for the presence of the same exon 3 mutation in different families. The haplotype of the allele harboring the exon 3 mutation in Family B was identical to that of Family A, also harboring the exon 9 mutation on the same allele, indicating that the latter occurred subsequently. The ancestral wild-type *TSHR* was present in Family B, suggesting that the mutation in exon 3 was also new in the history of that population.

Conclusions: It is more likely that two consecutive mutational events occurred on the ancestral wild-type allele instead of a recombination bringing exon 3 and exon 9 mutations together on the same allele. New mutational events contribute to the high prevalence of *TSHR* mutations in this population in addition to a founder effect and limited gene pool due to inbreeding.

Introduction

RESISTANCE TO THYROID STIMULATING HORMONE (RTSH; OMIM #275200) is a syndrome of reduced sensitivity to TSH, characterized by elevated serum TSH levels, absence of goiter, and normal or low levels of thyroid hormones (1). Inactivating *TSHR* gene mutations are the most common causes for RTSH. The *TSHR* gene (MIM #603372) is located on the long arm of chromosome 14 (14q31) (2,3); it comprises 10 coding exons and encodes a G-protein-coupled receptor with the classical seven transmembrane domains. Loss-of-function mutations in the *TSHR* gene lead to RTSH by multiple mechanisms, that is, reduced/absent receptor expression on the cell surface, impairment of ligand binding, and inability of receptor to get activated and act onto G-proteins. The consequences are variable depending on the severity of the functional impairment of the mutant gene. Homozygotes or

compound heterozygotes may have compensated hypothyroidism (only mild elevation of serum TSH concentration), or frank hypothyroidism that may be mild, moderate, or severe. Some heterozygotes may also manifest only an elevated level of serum TSH.

RTSH was considered to be a rare condition. However, in a recent large population-based Japanese study, the prevalence of monoallelic *TSHR* mutation was found to be as high as 9.4% in patients found to have high TSH at birth (4). Based on Hardy-Weinberg distribution, the expected frequency of heterozygous *TSHR* mutation was estimated to be 0.6% in the general Japanese population. Cordella and colleagues found an even higher prevalence of *TSHR* mutations, 22.9% in a population of young subjects with nonautoimmune subclinical hypothyroidism (5).

From the large population-based investigation in Japan, one particular mutation, c.1349G>A (p.R450H), is due to a

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founder effect and explains in part the high prevalence of *TSHR* mutation in Japan (4). Although the occurrence of genetic diseases in an isolated population suggests a founder effect, in many cases the increased frequency is due to more than one mutation (6). Our laboratory has identified six *TSHR* mutations—c.202C>T (p.P68S), c.267G>T (p.L89L), c.269_270AG>CT (p.Q90P), c.790C>T (p.P264S), c.1825C>T (p.R609X), and c.1957C>G (p.L653V)—in consanguineous Arab Muslim communities living in Northern Israel (7–10). The frequency of *TSHR* mutations was 4.3% in one tribe previously not known to have thyroid disease (9). Several hypotheses such as chance phenomenon, migration of families with affected patients, high mutation rates, digenic inheritance, and selective advantages to carriers have been proposed (6).

We previously reported a kindred of 30 individuals from the same Arab population (referred herein as Family A), in which three nucleotide substitutions were identified in exon 3 of the *TSHR* producing p.[L89L; Q90P] and a mutation in exon 9 of the same allele producing p.P264S (10). Another family of 12 individuals, reported herein (Family B), belonging to a different tribe harbors the identical *TSHR* mutations in exon 3 but not that in exon 9. The aim of this study was to determine whether the mutations in exon 3 of the *TSHR* gene in these two families have common ancestral origin.

Materials and Methods

Studies were approved by the Institutional Review Boards of the University of Chicago and written informed consents were obtained.

Index family (Family B)

The proband (subject III-2) was the second child of a consanguineous Arab Muslim couple, born after an unremarkable pregnancy and labor. This family, originating from Syria, resides in the central Israel. Newborn screening for congenital hypothyroidism revealed TSH of 31.4 mIU/L and a total thyroxine (T_4) of 8.5 μ g/dL that is below 10th percentile. A serum TSH obtained a few days later was 8.66 mIU/L (normal range, 1.7–9.1) and free T_4 (FT₄) 12.6 pmol/L (normal range, 11–18.8). At the age of 2 months, he was referred to a pediatric endocrinologist because of hyperthyrotropinemia. Physical examination revealed no sign of hypothyroidism. Laboratory tests found hypothyroidism: TSH 12.7 mIU/L (normal range, 0.35–4.2), FT₄ 10.6 pmol/L (normal range, 11.4–20.9), and free 3,3',5-triiodothyronine (FT₃) 3.4 pmol/L (normal range, 3.5–6.5). Antibodies to thyroperoxidase (TPO) and thyroglobulin (Tg) were absent. Treatment with 5 μ g/kg/day levothyroxine (L-T₄) was initiated at the age of 2 months. At the age of 3 years, the thyroid gland had normal size and position that was found by ultrasonography. ^{99m}Pertechnate thyroid scintigraphy showed normal uptake in a eutopic thyroid gland. After discontinuation of L-T₄ at 3 years of age, the serum TSH rose to a level of 22.1 mIU/L with a normal FT₄ concentration of 12.6 pmol/L. Clinical assessment to date reveals normal growth and development.

His brother (subject III-4) had a similar pattern of thyroid function tests without stigmata of hypothyroidism. He received L-T₄ of 5 μ g/kg/day since the age of one month and his TSH level was normal. Serum Tg levels before and during L-T₄ therapy were 42.2 and 10.7 ng/mL. His growth and de-

velopment have been normal. Their parents and siblings were clinically and biochemically euthyroid (Fig. 1). The proband and his brother were found to be compound heterozygotes for *TSHR* gene mutations. In one allele an AG to CT substitution in exon 3 (c.269_270AG>CT) replaces the normal glutamine at codon 90 with a proline (p.Q90P) and the other allele had a C to G transversion in exon 10 (c.1957C>G) that results in replacement of the normal leucine with a valine at codon 653 (p.L653V). A silent mutation due to a G to T transversion in codon 89 (c.267G>T) that results in no change of leucine (p.L89L) was also found in the allele harboring the exon 3 *TSHR* gene mutation. These mutations were reported before in Family A (10) and Family C (8,9) (see Family A and Family C below). These findings prompted sequencing of the entire *TSHR* gene in all family members.

Family A

Three index cases from an extended kindred of Arab Muslims living in Northern Israel presented with hyperthyrotropinemia or congenital hypothyroidism. We reported this family to have the same three nucleotide substitutions in exon 3 as Family B, but also had a mutation in exon 9 (p.P264S) on the same allele (10).

Family C

The proband, also from Arab Muslim descent living in Northern Israel, had compensated hypothyroidism. She was homozygous for a missense *TSHR* gene mutation, p.L653V (8,9). This mutation was presented in one allele of the proband in Family B.

Thyroid function tests

Total T_4 , total T_3 , and TSH were measured by chemiluminescence immunometric assays using the Elecsys Automated System (Roche Diagnostics, Indianapolis, IN). Total rT₃ (reverse T_3 or 3,3,5'-triiodothyronine) was measured by a commercial RIA (Adaltis Italia, Bologna, Italy) and Tg by an in-house RIA (11). The FT₄ index and FT₃ index were calculated as the product of the total serum concentrations of each iodothyronine and the normalized resin T_4 uptake ratio. Antibodies against Tg and TPO were measured by passive hemagglutination (Fujirebio, Inc., Tokyo, Japan).

Molecular analysis

Genomic DNA was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA) according to the manufacturer's instruction. For sequencing, all 10 coding regions and flanking introns of the *TSHR* gene were amplified using primers and thermocycler setting similar to those previously described (12). The resulting polymerase chain reaction products were visualized on 1.8% agarose gel before direct automated sequencing.

Several polymorphic markers in exonic and intronic regions of the *TSHR* gene (rs2239610, rs17111401, rs77924349, rs2075179, rs56751666, and rs1991517) were used for haplotyping. Three standard polymorphic microsatellite markers (D14S74, D14S606, and D14S616) spanning about 6.54 Mb or 5.3 cM (Marshfield map) around the *TSHR* locus were also used for haplotyping as detailed previously (13). The rs10141077 and rs77401871, single-nucleotide polymorphisms

FIG. 1. Pedigree and haplotypes of members of the Family B. Square symbols indicate men, circles women, and lozenges any gender. Double lines between spouses indicate consanguinity. The genotype of each subject is indicated within the symbol, as identified in the legend above the pedigree. Results of thyroid function tests are aligned with each symbol and were obtained on no hormonal replacement except for subjects III-2 and III-4 who were on 5 µg L-T₄/kg body weight (indicated by asterisk). Roman numerals to the left of the pedigree indicate the generation and numerals on the right of each symbol indicate individual family member. Subjects were tested for the polymorphic markers. The haplotypes are shown below each symbol and outlined with solid or dashed line to trace the inheritance of the different alleles harboring the mutations. The letter N indicates the ancestral allele without mutation.

(SNPs) that were identified during direct sequencing of D14S606, were included for analysis. Nucleotide numbering for mutations and variants uses cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference human sequence, NM_000369.2.

Statistical analyses

Data of thyroid function tests expressed as mean ± SD were analyzed by two-tailed Student's *t*-test for unpaired observations.

Results

Genetic and clinical results

Two previously described loss-of-function mutations of *TSHR* gene, p.Q90P and p.L653V, were identified in the proband on different alleles. In addition, a silent mutation p.L89L was found on the same allele as the p.Q90P mutant allele and identical to that of Family A reported previously (10). Of the 12 family members studied, 9 were found to harbor *TSHR* gene mutations (Fig. 1). The proband and his brother (subjects III-2 and III-4) were compound heterozygous for p.[L89L;Q90P], inherited from father, and p.L653V, inherited from mother. Father, two paternal aunts, and oldest

brother of the proband (subjects II-1, II-3, II-6, and III-1) were heterozygous for p.[L89L;Q90P]. Mother, maternal aunt, and sister of the proband (subjects II-2, II-8, and III-3) were heterozygous for p.L653V.

The intronic variant c.880+3A>G identified in the alleles of subjects II-2, II-7, and III-1 was presumed not to produce exon skipping. A guanine is found at position +3 in 35% of intron donor sites, known as consensus sequence (14). Also heterozygotes for this variant had normal serum TSH concentration.

The serum TSH concentrations of subjects heterozygous for the *TSHR* gene mutations p.L89L;Q90P ($n=4$, TSH 3.08 ± 1.42 mIU/L) and heterozygous for p.L653V ($n=3$, TSH 2.4 ± 0.45 mIU/L) were not statistically different than the TSH concentrations of family members with wild-type (WT) alleles ($n=3$, TSH 4.23 ± 4.02 mIU/L). Three family members with *TSHR* gene mutations were receiving L-T₄ treatment: two (III-2 and III-4) were compound heterozygous and one (II-3) was heterozygous for p.[L89L;Q90P] and had anti-Tg antibodies.

Haplotype analysis

Analysis of microsatellite markers (D14S74, D14S606, and D14S616) and two SNPs (rs10141077 and rs77401871) spanning 5.3 cM of the *TSHR* locus, known intragenic

polymorphic markers (rs2239610, rs17111401, rs77924349, rs2075179, rs56751666, and rs1991517), and a variant of intron 9 donor splice site (c.880+3A>G) residing in *TSHR* gene generated a common haplotype among individuals having p.[L89L;Q90P] allele in Family B. In subjects II-1 and II-3 heterozygous for p.[L89L;Q90P], the WT allele had the same haplotype (allele N, see Fig. 1) except for the exon 3 mutation and D14S616 located 3.66 Mb downstream from the exon 3 mutation site. This later difference could be from recombination or mutation because microsatellite markers have higher mutation rates than SNPs. Furthermore, subjects from Family A, with exon 3 and exon 9 mutations on the same allele, had an identical allele as subjects from Family B with exon 3 mutation, except for the exon 9 mutation and D14S616. These haplotypes support the notion that exon 3 and exon 9 mutations developed on this ancestral WT *TSHR* allele identified in Family B (Table 1) through consecutive mutational events. In addition there is evidence of common ancestry for the allele harboring the p.L653V mutation of Family B with the allele harboring the same mutation in Family C as they share identical haplotypes except for marker D14S606 (Table 1).

Discussion

Data on the prevalence of inactivating mutations in the *TSHR* gene in patients with congenital hyperthyroidism or nonautoimmune hyperthyrotropinemia are variable, from 2% to 40% depending on selection criteria of the population studied (9,15–22). Population-based genetic study in Japan showed that the *TSHR* mutations are not an uncommon cause of RTSH (4). However, a founder mutation that accounts for about 70% of mutants is responsible for the high prevalence of *TSHR* mutations in this population.

To date, six inactivating mutations have been found in Israeli Arab Muslim patients with congenital hypothyroidism belonging to three large extended kindreds (7,9,10). With the exception of p.R609×, these mutations were found only in this population. The p.R609× mutation was also reported in a consanguineous family of Turkish origin (23). The combina-

tion of common ancestry and intense cultural consanguinity accounts for a high inbreeding rate in these populations. Our previous study showed a prevalence of 4% in a control population of the same origin with no known thyroid diseases (9). In the current study, we demonstrated founder effect for the p.[L89L;Q90P] and p.L653V mutations.

Furthermore, two consecutive mutational events were demonstrated by haplotyping of the *TSHR* locus. This supports the hypothesis that the high prevalence of *TSHR* mutations in this Arab Muslim population is caused not only by a founder effect and limited gene pool due to breeding, but also by new mutation events. From the haplotyping study, we identified a specific allele carrying the mutants (except for the difference in the distal markers that could result from recombination after several generations). The allele with the exon 3 mutation (p.[L89L;Q90P]) was found to be identical to an ancient WT *TSHR* allele also present in Family B with all genetic markers on these alleles being identical, except for the exon 3 mutation. Moreover, our data suggest that the exon 9 (p.P264S) mutation occurred on this already mutated allele to generate the allele present in Family A that harbors both exon 3 and exon 9 mutations. This is supported by the fact that the other genetic markers on these alleles, including the distal markers rs1991517, rs10141077, rs77401871, and D14S606, are identical and not different as it would be expected if the mutation p.P264S occurred by recombination. The diversity of WT allele haplotypes strengthens the hypothesis of common ancestral allele.

Many different inactivating *TSHR* gene mutations including missense, nonsense, frameshift deletions/insertions, and splice site mutations have been documented to be responsible for RTSH. These loss-of-function mutations are found throughout the receptor sequence, without clear localization to hot spots such as CpG regions (24). Whether mutations in the *TSHR* gene may impart a selective advantage to carrier subjects (6,25) will require more studies to explore this hypothesis.

Mean TSH concentrations in the subjects with monoallelic inactivating *TSHR* mutations, p.[L89L;Q90P] and p.L653V,

TABLE 1. COMPARISON OF THE ANCESTRAL WILD-TYPE AND MUTANT *TSHR* ALLELES IN DIFFERENT FAMILIES

Family	Mb ^a	Ancestral allele B	Ex3 Mut B	Ex3 and 9 Mut A	Ex10 Mut B	Ex10 Mut C
D14S74	78.65	5	5	5	8	8
c.170+63G>C (rs2239610)	<i>TSHR</i>	G	G	G	G	G
c.171-80T>A (rs17111401)	(81.42–81.61 Mb)	A	A	A	T	T
p.[L89L;Q90P]		WT	Mut	Mut	WT	WT
c.318-35C>T (rs77924349)		C	C	C	C	C
c.561T>C (rs2075179)		T	T	T	T	T
c.615-19_615-18ins4 (rs56751666)		1	1	1	1	1
p.P264S		WT	WT	Mut	WT	WT
c.880+3A>G		A	A	A	A	A
p.L653V		WT	WT	WT	Mut	Mut
c.2181G>C (rs1991517)		C	C	C	C	C
rs10141077, rs77401871	82.25	T	T	T	T	T
D14S606	82.25	10	10	11	10	9
D14S616	85.19	9	8	9	9	9

Previous reported *TSHR* mutations in Arab population, c.202C>T (p.P68S) and c.1825C>T (p.R609×), were not found in these families.

^aMarker location on the reference human sequence (NM_000369.2).

Ex, exon; **Mut**, mutation; WT, wild-type.

and with the WT TSHR were not significantly different. However, the sample size is too small to draw a definite conclusion. Additional family members refused to participate in the study. The subjects compound heterozygous for the two mutations had compensated hypothyroidism. These results are compatible with high residual function of the mutant TSHRs as shown by functional analyses in the previous studies (9,10). This indicates that these two mutations have a mild effect on TSHR inactivation when present in heterozygotes or even together in compound heterozygotes. Our previous study showed that heterozygotes for the p.L653V allele had significant higher mean TSH level than family members with WT allele only (9). Thyroid function tests can vary between different individuals affected with the same mutation or among members of the same family. These findings suggest that environmental factors (e.g., thyroid hormone requirement, iodine supply, or acquired thyroid insults) as well as yet unknown genetic modifiers may account for this difference in the phenotype.

In conclusion, we propose that two consecutive mutational events occurred on an ancient WT allele generating the allele with both exon 3 and exon 9 mutations, instead of a recombination event that brought exon 3 and exon 9 mutations together on the same allele. This indicates that new mutational events contribute to the high prevalence of *TSHR* mutations in this population in addition to a founder effect and limited gene pool due to inbreeding.

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Author Disclosure Statement

S.R. consults for Quest Diagnostics. The remaining authors have no competing financial interests.

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