TTF-2/FOXE1 gene polymorphisms in Sicilian patients with permanent primary congenital hypothyroidism*

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ABSTRACT. Thyroid transcription factor-2 (TTF-2/FOXE1) is a polyalanine domain protein that regulates thyroid embryogenesis, but very few patients with permanent primary congenital hypothyroidism (pCH) harbor germline mutations of this or other transcription factors that are involved in thyroid development that might explain the etiology of pCH. Variations within the polyalanine tract are found in a variety of genes and are often reported in association with malformation syndromes; pCH is frequently associated with thyroid malformations and extra-thyroidal malformations. Therefore, in this study we investigated whether alanine (Ala) length polymorphisms and non-polymorphic mutations of the *TTF-2* gene in pCH patients might be involved in the pathogenesis of pCH. The entire coding region of the *TTF-2* gene was analyzed in 57 Sicilian patients and 142 healthy controls. We found that the homozygous Ala14 polymorphism (Ala14/14) was less frequent in the pCH group than in the controls. In contrast, significantly more pCH patients than controls harbored the Ala16 polymorphism (Ala16/16 and Ala14/16). However, neither the Ala14/14 nor the Ala16 polymorphism was related to extra-thyroidal malformations. Two of the 57 patients carried Ala11/14 and Ala12/14, and one Ala14/14 patient also had the silent polymorphism 387 C/T (Leu129Leu). Other than known polymorphic variants we found no mutation in the *TTF-2* gene. Therefore, this study demonstrates that mutations in the *TTF-2* gene are rare in pCH patients and suggests that variations in the length of the Ala-tract could at least partially explain the etiology of pCH but not that of extra-thyroidal malformations.
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INTRODUCTION

Congenital hypothyroidism (CH) is the most frequent endocrine disorder in neonates, occurring in between 1 in 2,500 and 1 in 3,000 live births. CH may be either permanent or transitory and results in severe neurodevelopmental impairment if untreated (1). A high prevalence of thyroid dysgenesis in Hispanics and Caucasians, a predominance of thyroid dysgenesis in girls, and a high prevalence of associated malformations implicate genetic factors in the etiology of CH (2). The leading cause of permanent CH is thyroid dysgenesis (85% of cases). The remaining cases are due to thyroid dyshormonogenesis (~10%) and hypothalamus-pituitary abnormalities (~5%) (3, 4). Consistent with the dysgenetic nature of permanent primary CH (pCH), patients with the condition have a relatively high prevalence of extra-thyroidal malformations, mainly cardiac (2, 5-9).
Thyroid dysgenesis, which is familial in up to 2% of cases, is a collective term for a variety of conditions, namely agenesis, hemiagenesis, hypoplasia and ectopy (10). Results obtained from gene knockout animal models suggest that thyroid dysgenesis involves the thyrotropin receptor and a number of transcrip-

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tion factors (TTF-1, *TTF-2*, PAX-8 and NKX2.5) that play a role in the morphogenesis, migration and growth of the thyroid (10, 11), but very few patients with thyroid dysgenesis-induced pCH have germline mutations of any of these genes that would account for the disorder (12-18). Thus, the pathogenesis of pCH remains largely unknown.

TTF-2/FOXE1 belongs to a family of transcription factors characterized by a forkhead/winged helix DNA binding domain. Members of this family are key regulators of embryonic pattern formation and regional specification (19). *TTF-2* is expressed in the human thyroid as well as the oropharyngeal epithelium and thymus (20). The targeted disruption of *TTF-2* in mice results in thyroid agenesis or thyroid ectopy and a cleft palate (10). Therefore, human athyrosis and ectopy may represent different degrees of severity of the same molecular defect. Indeed, a defect of the human *TTF-2* gene was first seen in two Welsh male siblings born with cleft palate, choanal atresia, spiky hair, bifid epiglottis (Bamforth syndrome), and pCH due to thyroid agenesis (15). In an unrelated family, two probands with pCH due to thyroid agenesis and with a milder phenotype were homozygous for another missense mutation of the *TTF-2* gene (16).

The human *TTF-2* gene is located on chromosome 9, contains a single exon and encodes a protein that consists of an N-terminal region, a highly conserved forkhead domain, a helical polyalanine tract, and a single C terminal tail (15, 19). The *TTF-2* polyalanine tract is polymorphic and has been reported to range from 11 to 19 alanine (Ala) residues (21-23). About 500 human proteins contain polyalanine domains, and about one third of them contain a minimum of seven consecutive Ala (24). The number of Ala residues in the tract can change depending on expansions or contractions of an Ala-encoding triplet (most frequently GCG). Above a threshold of 19 Ala residues, these proteins self-aggregate and form intracellular inclusions that ultimately lead to cell death (25). Polymorphic variations in the number of Ala residues have been identified in several transcription factors (24, 25), such as FOXL2 (blepharophimosis-ptosis-epicanthus inversus syndrome), SOX3 (infundibular hypoplasia and hypopituitarism), ARX (X-linked mental retardation and abnormal genitalia) and ZIC 2 (holoprosencephaly), and all have been associated with congenital malformation syndromes. Polymorphism of the polyalanine tract of *TTF-2* in pCH has been studied in only two series of patients, one in Japan (22) and one in Tuscany, Italy (26).

We hypothesized on the basis of these findings that changes in the length of the polyalanine stretch of *TTF-2* are involved in the pathogenesis of pCH. To test this hypothesis, we sequenced the *TTF-2* exon in both patients and controls to search for polymorphic and non-polymorphic variants of the gene and investigated whether Ala polymorphisms are related to the etiology of pCH or to pCH-associated extrathyroidal malformations.

MATERIALS AND METHODS

Patients

Our control group consisted of 142 healthy Sicilian infants. Our patient group consisted of 57 Sicilian infants with pCH (42 girls and 15 boys), which was detected by neonatal screening based on the measurement of TSH in a blood spot sampled on day 4 or 5 of life. TSH was measured by an immunoenzymatic assay (normal range: 1.0-30.0 mU/l). Measurements of TSH and free T_4 (FT_4) serum levels confirmed the diagnosis. The etiology of pCH was identified by thyroid ^{99m}Tc scintigraphy and ultrasonography. The etiologies were ectopy (no. 30), agenesis (no. 17) or hemiagenesis (no. 3) and hypoplasia (no. 7).

Malformations were defined according to Stevenson and Hall's criteria (27). Umbilical hernia, which is frequently observed as a reversible clinical sign in untreated CH patients, was not accounted in our study. Two premature infants with ductus arteriosus malformation were excluded from the study because of the high frequency of this condition in pre-term neonates. We also excluded patients with Down's syndrome or other chromosomal abnormalities as well as infants with central CH and three pCH patients with non-dysgenesis thyroid. Cardiac malformations were identified by conventional one- and two-dimensional echocardiography with Doppler and color flow mapping.

DNA extraction and amplification

We isolated genomic DNA from the peripheral blood lymphocytes of 57 pCH patients and 142 controls with the QIAmp DNA Blood Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's guidelines. The exon of the *TTF-2* gene was amplified by using previously described primers (28). Briefly, polymerase chain reaction (PCR) amplification was carried out in a final volume of 25 µl with 0.1 µg of genomic DNA, 25 pmol of each primer, 200 µM deoxy-nucleotide triphosphate, PCR buffer (10 mM Tris-HCl, 50 mM KCl), 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide and 1.0 U of *Taq* DNA polymerase (Applied Biosystems, Monza, Italy). A 7-min denaturation step at 95 C was followed by 30 cycles consisting of 50 sec at 95 C, 45 sec at the corresponding annealing temperatures and 1 min at 72 C and a final extension of 10 min at 72 C.

Genetic analysis and DNA sequencing

The entire coding region of the *TTF-2* gene was amplified and directly sequenced as previously reported (28). To study the allelic frequency of polyalanine stretch lengths, we amplified genomic DNA from the 57 pCH patients and 142 controls by using the following primers: forward 5'-TCA AGC GCT CGG ACC TCT CCA CCT A-3' and reverse 5'-CGG GCG ACG CCG CGG GGT AGT AGA C-3'. Differences in PCR-product lengths were analyzed on 3% Metaphor agarose gels (FMC Bioproducts, Rockland, ME, USA). Polymorphisms of polyalanine stretch lengths were confirmed by direct sequencing of the amplification products with an ABI Prism 310 genetic analyzer (Applied Biosystem, Foster City, CA, USA) by using the BigDye terminator v3.1 cycle sequencing kit (Applied Biosystem).

Statistical analysis

We used two-sided Fisher's exact test to compare Ala polymorphisms between infants with pCH and healthy Sicilian infants and to compare extra-thyroidal malformations between our pCH infants and patients from the Italian registry for congenital hypothyroidism (8). Differences were considered significant for levels of $p<0.05$. The odds ratios (OR) and corresponding 95% confidence interval (CI) are also provided.

RESULTS

Relationship between Ala polymorphisms and the etiology of pCH

As shown in Table 1, the most frequent Ala polymorphism in the group of pCH infants was homozygosity for a stretch of 14 consecutive Ala (Ala14/14). In fact, Ala14/14 was found in 38 pCH infants (67%), which is significantly fewer than the control group (89%; $p=0.0003$), and the difference remained significant even when the three dysgenetic subgroups were considered separately (Table 1). The next most frequent polymorphism in the pCH group, identified in 26% of infants, was heterozygosity for Ala14/16, and again there was a significant difference ($p=0.008$) compared with the controls (11%). Three other polymorphisms (Ala16/16, Ala12/14, or Ala11/14) were detected in a few patients, but none of these polymorphisms were detected in the controls ($p>0.05$).
The agenesis/hemiagenesis subgroup was associated with four of the five Ala-stretch polymorphisms identified (Table 1).
In addition to the Ala14/14 polymorphism, one patient with ectopy also carried the 387 CCT/CCT polymorphism (frequency 1.7%), which is silent, as it does not affect Leu in codon 129. Of note, however, this polymorphism was not found in the control group.

Relationship between Ala polymorphisms and extra-thyroidal malformations

The rate of patients with malformations in the ectopy, agenesis/hemiagenesis and hypoplasia subgroups was 23% (7/30), 25% (5/20) and 14% (1/7), respectively (Table 2). However, the frequency of cardiac malformations was higher in patients affected by ectopy (17%, 5/30) than in patients affected by agenesis/hemiagenesis (5%, 1/20). No patients with hypoplasia had cardiac malformations. The rate of patients with extra-thyroidal malformations in our series was greater than that reported for 1372 patients of the Italian registry for congenital hypothyroidism 22.8 *vs* 8.4%, $p=0.001$; OR=3.23 (95% CI 1.69-6.17), especially oral clefts (5 *vs* 0.4%; $p=0.004$; OR=12.65 (95% CI 3.08-51.93). However, the distribution of malformations among pCH etiologies was similar (ectopy, 54 *vs* 45%; agenesis/hemiagenesis, 38 *vs* 39%; hypoplasia 8 *vs* 5%; eutopic and normally sized thyroid, 0 *vs* 11%). Compared with the Italian registry, our data showed a higher rate of cardiac malformations, although the difference did not achieve statistical significance (11 *vs* 4.8%, $p=0.06$; OR=2.32 (95% CI 0.96-5.62), but the ratio of ASD:VSD:pulmonary valve stenosis: tetralogy of Fallot was similar (3: 2: 1: 1 *vs* 3: 2: 1: 1).
The rate of the Ala14/14 polymorphism did not differ between patients with (77%, 10/13) or without (64%, 28/44) extra-thyroidal malformations [$p=0.51$; OR=1.91 (95% CI 0.46-7.95)]. Similarly, the rate of the Ala14/16 polymorphism did not differ between patients with (23%, 3/13) or without (27%, 12/44) extra-thyroidal malformations [$p=1.0$ OR=0.80 (95% CI 0.19-3.4)].

Table 1 - *Prevalence of the polyalanine tract length polymorphisms of the thyroid transcription factor-2 (TTF-2/FOX1) gene in infants with primary congenital hypothyroidism (pCH) and in controls.*

	Controls	All etiologies			Thyroid ectopy			Thyroid agenesis/ hemiagenesis			Thyroid hypoplasia		
	(No. 142)	(No. 57)			(No. 30)			(No. 20)			(No. 7)		
		No.	OR (95% CI)	*p*-value	No.	OR (95% CI)	*p*-value	No.	OR (95% CI)	*p*-value	No.	OR (95% CI)	*p*-value
ALA 14/14	127 (89%)	38 (67%)	0.24 (0.11, 0.51)	0.0003	21 (70%)	0.28 (0.11, 0.71)	0.02	13 (65%)	0.22 (0.08, 0.64)	0.01	4 (57%)	0.16 (0.03, 0.77)	0.04
ALA 14/16	15 (11%)	15 (26%)	3.02 (1.36, 6.70)	0.008	8 (27%)	3.08 (1.17, 8.12)	0.03	5 (25%)	2.82 (0.90, 8.87)	0.08	2 (29%)	3.39 (0.60, 19.01)	0.18
ALA 16/16	0 (0%)	2 (4%)		0.08	0 (0%)	--	--	1 (5%)	--	0.12	1 (14%)	--	0.047
ALA 12/14	0 (0%)	1 (2%)		0.29	1 (3%)	--	0.17	0 (0%)	--	--	0 (0%)	--	--
ALA 11/14	0 (0%)	1 (2%)		0.29	0 (0%)	--	--	1 (5%)	--	0.12	0 (0%)	--	--

Table 2 - *Polyalanine tract length polymorphisms of the* TTF-2/FOXE1 *gene and extra-thyroidal congenital malformations in 57 Sicilian patients, stratified on the basis of etiology of primary congenital hypothyroidism (pCH).*

Patients and pCH etiology	Polyalanine tract length polymorphisms	Extra-thyroidal malformations
Thyroid ectopy		
1	14/14	Partial ASD
2	14/14	VSD
3	14/14	Tetralogy of Fallot
4	14/14	ASD
5	14/14	Bifid spine
6	14/14	Foot syndactyly + hypogonadism
7	14/16	Pulmonary valve stenosis
8-14	14/16	Absent
15-29	14/14	Absent
30	12/14	Absent
Thyroid agenesis/hemiagenesis		
1	14/14	Hypertelorism with bilateral epicanthus
2	14/14	Cleft palate
3	14/14	Cleft palate + cleft lip
4	14/16	Septo-optical dysplasia
5	14/16	ASD + VSD and cleft palate
6-8	14/16	Absent
9-18	14/14	Absent
19	11/14	Absent
20	16/16	Absent
Thyroid hypoplasia		
1	14/14	Ectopic ureterocele
2-4	14/14	Absent
5,6	14/16	Absent
7	16/16	Absent

ASD: atrial septal defect; VSD: ventricular septal defect. Malformations were defined according to Stevenson and Hall (27). See "Materials and Methods" for further details.

DISCUSSION

In this study of 57 Sicilian infants with pCH, we detected no mutations of the *TTF-2* gene other than an already known silent single nucleotide polymorphism and polymorphic expansions or contractions of the alanine-encoding nucleotide triplets that lead to variations of the length of Ala tracts within the polyalanine domain of the *TTF-2* gene product. Our finding of polymorphisms only is in accordance with the studies from Japan (22) and from Tuscany, Italy (26) (Table 3). In all three studies, the Ala12/14 genotype occurred in 2% of pCH patients but not in any of the controls. The Ala11/14 genotype occurred in up to 2% of pCH patients, but not in any of the controls. Unlike the Japanese study, Ala14/14 and Ala14/16 were the two most frequent genotypes in the two Italian studies. Thus, in Italian pCH patients, Ala14/14 is underrepresented by a factor of 0.8 compared with the controls, whereas Ala14/16 is overrepresented by a factor of 2 to 3. In Japanese pCH patients *vs* the controls, Ala14/14 is equally represented whereas Ala 14/16 is underrep-

Table 3 - *Prevalence of polymorphisms of the TTF-2 gene in patients with primary congenital hypothyroidism (pCH) and in controls in the present study compared with two previous studies (22, 26).*

	pCH patients			Controls			Patients: controls ratio		
	Sicily (No. 57)	Tuscany (No. 51)	Japan (No. 46)	Sicily (No. 142)	Tuscany (No. 51)	Japan (No. 101)	Sicily	Tuscany	Japan
Ala 14/14	67%	72%	94%	89%	86%	95%	0.8 : 1	0.8 : 1	1 : 1
Ala 14/16	26%	24%	2%	11%	7%	4%	2.4 : 1	3.4 : 1	0.5 : 1
Ala 16/16	3.5%	2%	0	0	7%	1%	3.5 : 0	0.3 : 1	0 : 1
Ala 12/14	2%	2%	2%	0	0	0	2 : 0	2 : 0	2 : 0
Ala 11/14	2%	0	2%	0	0	0	2 : 0	0 : 0	2 : 0
387 C/T (Leu129Leu)	2%	2%	2%	0	0	0	2 : 0	2 : 0	2 : 0
519 A/T (Ala173Ala)	0	0	1%	0	0	0	0	0	1 : 0
723 C/T (Pro241Pro)	0	0	5%	0	0	10%	0	0	0.5 : 1
834 T/C (Ser278Ser)	0	2%	3%	0	0	5%	0	2 : 0	0.6 : 1

Concerning pCH etiology, in the studies from Tuscany (26) and Japan (22) thyroid ectopy accounted for 37 and 33%, agenesis/hemiagenesis for 57 and 11%, hypoplasia for 4 and 13%, and thyroid *in situ* for 2% and not specified. The etiology of pCH is reported as "undetermined" in 43% (20/46) of the Japanese patients.

resented by a factor of 0.5. Our study and the Tuscan study also identified a similar narrow spectrum of silent polymorphims (387 C/T detected in both studies and 834 T/C detected in the Tuscan study only). The prevalence of the 387 C/T silent polymorphism was the same in the three studies, namely, 2% in pCH patients and 0% in healthy controls, whereas the prevalence of 834 T/C was higher in Japanese than in Tuscan patients. This polymorphism was also found in the healthy Japanese population. Therefore, there is overall agreement between the two Italian series despite a difference in the distribution of pCH etiology (Table 3, footnote). However, the rates of thyroid ectopy, agenesis/hemiagenesis and hypoplasia (50, 33 and 12%, respectively) in our series are not very far from those of the Italian registry for congenital hypothyroidism (51, 32 and 5%, respectively) (8).

Unfortunately, neither Hishinuma et al. (22) nor Tonacchera et al. (26), the investigators in the studies in Japan and Tuscany, provided data about extra-thyroidal malformations in their patients. The rate of extra-thyroidal malformations (22.8%) in pCH patients is at the upper end of the range reported in other studies (5 to 24%) (2, 5-9) and greater than that reported in the Italian registry for congenital hypothyroidism (8%) (8). We found that pCH patients with associated extra-thyroidal malformations carried either Ala14/14 or Ala14/16. However, patients with and without malformations were equally represented in both genotypes.

In our study, any change from the Ala14/14 genotype appeared to favor the phenotypic expression of pCH. This predisposition occurred regardless of whether an allele displayed a reduction (–3Ala→ Ala11; –2Ala→ Ala12) or an increase (+2Ala→ Ala16) in the number of consecutive Ala residues. Only data relating the transcriptional activity of the two forms of *TTF-2*/FOXE1 could definitely confirm our data.

Interestingly, it was recently demonstrated that the *TTF-2* polyalanine tract showed marked variation between premature ovarian failure (POF) patients and normal controls. The frequency of the 14 Ala allele was significantly less in POF patients than in controls and the frequency of the 16 Ala was significantly more in POF patients than in controls, suggesting that variation in the polyalanine tract predisposes to POF (23).

The mechanism by which one or more polymorphic variants of a protein containing a poly-Ala domain lead to a congenital syndrome is unknown. Changes in the number of consecutive Ala within the poly-Ala stretch probably affect the level of interaction of the transcription factor protein with DNA and/or other transcription factor(s) with which the poly-Ala protein interacts (29). This is in line with the lower proportion of pCH patients bearing the Ala14/14 polymorphic *TTF-2* variant compared with healthy controls. In other words, the

Ala14/14 variant exerts a protective effect toward pCH and particularly toward thyroid dysgenesis.
However, it is possible that certain alleles, or certain alleles in combination, are linked to a higher risk of thyroid complications. The hypothesis that the Ala-tract polymorphisms may be in linkage disequilibrium with other genetic variants should be considered. Such polymorphisms are not likely to alter gene structure or function, so they might not be directly related with change in phenotype but may act directly or indirectly with other DNA sequence variants (30). Loci in linkage disequilibrium are generally physically close; however, genetic markers that are immediately adjacent to each other on a chromosome might be independent, whereas those that are distant might be highly correlated (31).
In summary, other than known polymorphic variants, we found no mutations in the *TTF-2* gene. Furthermore, significantly fewer pCH patients than controls harbored the most frequent poly-Ala length polymorphism (Ala14/14). Consequently, one could hypothesize that this *TTF-2* variant confers a protective effect for pCH, but not for extra-thyroidal malformations. In contrast, significantly more pCH patients than controls harbored the second most frequent poly-Ala length (Ala14/16), which would suggest a heightened risk for pCH, but no influence on extra-thyroidal malformations. Ethnicity-related deviations from this schema are certainly possible. This study demonstrates that mutations in the *TTF-2* gene are rare in pCH patients and suggests that variations in the length of the Ala tract could, at least in part, explain the etiology of pCH and supports the concept that pCH is a multigenic disease.

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