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ORIGINAL INVESTIGATION

Polymorphic length of FOXE1 alanine stretch: evidence for genetic susceptibility to thyroid dysgenesis

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Abstract Familial cases of congenital hypothyroidism from thyroid dysgenesis (TD) (OMIM 218700) occur with a frequency 15-fold higher than by chance, *FOXE1* is one of the candidate genes for this genetic predisposition and contains an alanine tract. Our purpose is to assess the influence of length of the alanine tract of *FOXE1* on genetic susceptibility to TD. A case–control association study (based on 115 patients affected by TD and 129 controls genotyped by direct sequencing) and transmission disequilibrium testing (TDT) analyses were performed. The transcriptional activities of FOXE1 constructs containing 14 or 16 alanines were also studied. In the case–control association study, the 16/16 and 16/14 genotypes were inversely associated with TD (OR = 0.39, 95%CI = 0.22–0.68, $P = 0.0005$), strongly suggesting that the presence of 16 alanines in the tract protect against the occurrence of TD. This association was stronger in the subgroup of patients with ectopic thyroid (OR = 0.28, 95%CI = 0.13–0.58, $P = 0.00015$). The protection was confirmed by the TDT analysis performed in 39 trios ($\chi^2 = 4.3$, $P = 0.0374$). Alternatively, the presence of the 14/14 genotype is associated with an increase risk of TD (OR = 2.59, 95%CI = 1.56–4.62, $P = 0.0005$). The expression studies showed that the transcriptional activities of FOXE1 with 16 alanines were significantly higher (1.55-fold) than FOXE1 containing 14 alanines ($P < 0.003$), while the nuclear localisation of the proteins was not affected. We conclude that FOXE1 through its alanine containing stretch modulates significantly the risk of TD occurrence, enhancing a mechanism linking an alanine containing transcription factor to disease.

Keywords Polyalanine · FOXE1 · Thyroid dysgenesis · Genetic susceptibility

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Introduction

Abnormalities in thyroid development (thyroid dysgenesis, TD, which includes ectopic gland and thyroid agenesis or athyreosis) are the main causes of congenital hypothyroidism (CH), which is the most frequent congenital endocrine disorder, affecting one newborn in 3,500 in France (Castanet et al. 2001; Van Vliet 2005).

A familial component of TD has been described (Castanet et al. 2000, 2001) and mutations in five genes involved in thyroid development (*FOXE1, PAX8, TSHR, TTF1* and *NKX2.5*) have been shown to result in a minority of TD cases in humans (De Roux et al. 1996; Clifton-Bligh et al. 1998; De Felice et al. 1998; Devriendt et al. 1998; Mansouri et al. 1998; Vilain et al. 2001; De Felice and Di Lauro 2004; Sura-Trueba et al. 2005; Dentice et al. 2006).

Among TD-related genes, *FOXE1* encodes a transcription factor that contains a forkhead domain and a polyalanine (polyA) stretch of variable length and regulates the transcription of target genes such as thyroglobulin and thyroid peroxidase. Homozygous null mice embryos with targeted disruption of *FOXE1*, exhibit cleft palate and thyroid malformation consisting of either thyroid agenesis or thyroid ectopy suggesting a role for *Foxe1* in migration as well as in proliferation of the thyroid gland (De Felice et al. 1998; Parlato et al. 2004). In humans, only three different homozygous missense mutations within the forkhead domain have been reported so far to cause athyreosis associated with others malfomations such as cleft palate, bifid epiglottis and choanal atresia (Clifton-Bligh et al. 1998; Castanet et al. 2002; Baris et al. 2006).

Recently, expansions of trinucleotide repeats encoding polyA tracts have been recognized as the cause of at least nine diseases through the alanine containing proteins (Brown and Brown 2004). With the exception of PABPN1, which codes for a poly(A)-binding protein, the nine genes with alanine tract expansions described so far encode transcription factors that play important roles during development. Thus, all these polyA disorders result in early developmental effects, such as malformations of the brain or digits (Abu-Baker and Rouleau 2007). Recently, it has been shown that expansions of polyA tracts could result in protein misfolding and aggregation, which is toxic for the cells (Caburet et al. 2004). Moreover, alanine tracts of some transcription factors have been suggested to be involved in the ability to transactivate or repress the expression of target genes (Civitareale et al. 1994; Chadwick et al. 1997; Lavoie et al. 2003).

Polymorphism of the polyA tract of FOXE1 was reported for the first time by Macchia et al. with variable length from 12 to 17 alanines (Macchia et al. 1999). To date, three groups investigated the polyA tract length in TD populations and found polymorphism of this tract with at least 4 different lengths (11, 12, 14 and 16 alanines) (Hishinuma et al. 2001; Tonacchera et al. 2004; Santarpia et al. 2007). Among them, only one performed statistical analysis, suggesting that a FOXE1 variant could confer a protective effect for CH (Santarpia et al. 2007). However, it is noteworthy that frequency of alleles is quite different among the different control groups (Table 1), even among the same population (Macchia et al. 1999; Santarpia et al. 2007; Tonacchera et al. 2004). The mechanism by which polyA variants lead to CH remains to discuss. Only the Japanese group performed expression studies for tracts $\leq$14 alanines without finding any differences in terms of transcriptional activities, suggesting that the polymorphism of the polyA tract was not a cause of developmental defects of the human thyroid gland (Hishinuma et al. 2001).

These observations prompted us to investigate variations in FOXE1 polyA tract length in a large cohort of patients

Table 1 Distribution of FOXE1 polyA alleles in our cohort and in the literature (frequencies %)

Alleles (%)		Hishinuma et al. 2001	Tonacchera et al. 2004	Santarpia et al. 2007	Macchia et al. 1999	Watkins et al. 2006		
	French	Japanese	Tuscany	Sicilian	Italian	New Zealand	Slovenian	Total
Controls group								
11	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
12	0.0	0.0	0.0	0.0	2.0	0.4	1.3	0.6
14	65.1	97.0	88.7	94.7	54.0	67.5	66.2	67.2
16	33.7	3.0	11.3	5.3	40.0	30.0	23.0	28.4
17	1.2	0.0	0.0	0.0	4.0	0.0	0.0	0.0
19	0.0	0.0	0.0	0.0	0.0	2.1	9.5	3.8
Total, *n*	258	202	106	284	196	240	74	314
Patients group								
11	0.4	1.1	0.0	0.9				
12	1.3	1.1	1.0	0.9				
14	78.3	96.7	85.3	81.6				
16	17.4	1.1	13.7	16.7				
17	1.7	0.0	0.0	0.0				
19	0.9	0.0	0.0	0.0				
Total, *n*	230	92	102	114				

affected by TD in order to better elucidate the potential role of this tract in the genetic susceptibility to TD.

Materials and methods

Subjects and samples

This study included 115 unrelated patients affected with TD. Among them, 64 (55.7%) had ectopy, 35 (30.4%) athyreosis and 16 (13.9%) hypoplastic thyroid gland or hemiagenesis. They were recruited through our previous studies (Polak et al. 2004) and they were all French Caucasian.

For the case–control association study, 129 controls of French Caucasian background without history of thyroid disease were used. The sex ratio in both groups was not different (67.0% of women in cases, $n = 77$ vs. 57.4% in controls, $n = 74$; $\chi^2 = 2.4$, $P = 0.124$).

For the transmission disequilibrium test (TDT), parents of the affected cases were included when possible. A total of 39 trios have been analysed. We also performed separately the TDT for ectopic glands only (26 trios). Such an analysis could not be performed in patients with athyreosis only, due to a too small number of cases (10 trios).

Four pairs of monozygotic twins that were discordant for TD were also analyzed.

The study fulfilled the ethical legislation applied in France, it was approved by our local ethical committee. Parental consent was given on behalf of children. Genomic DNA was extracted from peripheral blood using standard procedures.

Alanine tract sequencing

Genomic DNA was amplified by PCR (GC rich Taq polymerase, Roche Diagnostics). The following primers were used: FOXE1 F4 5′GCGGAGGACATGTTCGAGA 3′ and FOXE1 R4 5′CGCGGGGTAGTAGACTGGAG 3′. The amplicons (234 to 258 bp; 11–19 alanines) were purified (Performa DTR Gel Filtration Cartridges, Edge BioSystems), directly sequenced with the "Big Dye Terminator v1.1 Cycle Sequencing" kit (Applied Biosystems), in an ABI Prism 3130 automatic sequencer (Applied Biosystems).

The remaining single coding exon of *FOXE1* gene was studied by Single Strand Conformation Polymorphism in the patients according to our published protocol (Castanet et al. 2002) and no abnormal migration profile, suggestive of a possible mutation was found.

Statistical analysis

Data were analysed using the Epi-Info software (version 6.04) and the SAS software (version 9.1, SAS Institute, Cary, NC, USA).

For the case–control study, the allelic and genotypic frequencies of the alanine tract were determined among the cases and controls, and compared with the χ^2 test. The Hardy-Weinberg Equilibrium (HWE) was tested in the control group and no significant deviation was observed. The strength of the association between each genotype and the disease was estimated by calculating the odds ratio (OR) with 95% confidence interval (95%CI). The genotype 14/14, being the most frequent both in cases and controls, was taken as reference for the calculations. We also tested the strength of the association between our marker and TD according to the gender and to the etiological type of TD.

To validate the case–control association study, a TDT was performed and calculated "by hand". The TDT enables to test the linkage between the marker locus and a disease susceptibility locus when an association has been found by a case–control study. This test is based on triads (affected child + parents) in which at least one parent is heterozygous for the marker. It allows to determine the allele of interest which has been more frequently transmitted by the parents to their affected child (Spielman et al. 1993). We had only 3 trios with both parents and child and we did not used them for the test because they were heterozygotes and not informative.

Plasmid and construction

For the functional studies, we used the FOXE1 expression vector (pFlagCMV-hFOXE1) described previously (Clifton-Bligh et al. 1998), which encodes 14 alanines (given by Chatterjee). To compare the functional consequences of the 14 and 16 containing alanines tracts, 2 additional alanines were introduced by site-directed mutagenesis (QuickChange Site-directed Mutagenesis Kit, Stratagene) using sense primer 5′-CGCCGCCGCCGCAGCAATCTT CCC-3′ and antisense primer 5′-GGGAAGATTGCTGCGG CGGCGGCG-3′ according to the manufacturer's protocol. The construct was validated by DNA sequencing.

Transient transfection

For functional studies, the 293A cells lines (Invitrogen) were grown in DMEM supplemented with 10% fetal bovine serum (Biowest) and 1% penicillin and streptomycin (Invitrogen).

For luciferase and galactosidase assays, a reporter gene containing FOXE1, TTF1 and PAX8 binding sites for the thyroid thyroglobulin promoter (given by Refetoff) upstream luciferase was used and has been described previously (Pohlenz et al. 2002). We plated the 293A cells at a density 2×10^5 per well in 24-well 24 h before transfection. We then transiently co-transfected cells with 520 ng of reporter gene, 260 ng of pFlagCMV-hFOXE1 (encoding 14

or 16 alanines), 130 ng of pcDNA3-hPAX8 (given by Vassart), 130 ng of pcDNA3-hTTF1 (from the pSG5-hTTF1 given by Refetoff) and 150 ng of plasmid BosβGalactosidase (given by Chatterjee). Co-transfection was carried out by a 6 h exposure to 3 µl of lipofectamine in Opti-Mem medium (Invitrogen). We chose to co-transfect this combination of transcription factors as it has been demonstrated that interactions between them (Perrone et al. 2000; Di Palma et al. 2003) and their simultaneous expressions are unique to thyroid follicular cells (Sura-Trueba et al. 2005).

For western blot, 5×10^6 cells were plated in a 100-mm diameter culture dish 24 h before transfection. Cells were then co-transfected with 4 µg pcDNA3-hPAX8, 4 µg pcDNA3-hTTF1 and 4 µg pFlagCMV-hFOXE1, using 30 µl lipofectamine.

For immunocytochemistry, 4×10^4 cells per well were plated in Lab-tek chamber slides (Nunc) 24 h before transfection. Cells were then transfected with 400 ng of pFlagCMV-hFOXE1 (encoding 14 or 16 alanines) or of pCAGGS-PHOX2B-IRES-EGFP (encoding 20 or 33 alanines given by Lyonnet) using 1.5 µl lipofectamine. As additional controls, cells were also transfected with empty plasmid (without FOXE1 and PHOX2B) (data not shown).

Luciferase and galactosidase assays

For luciferase assays, the transiently co-transfected 293A cells were harvested after 48 h and luciferase and beta-galactosidase assays were performed. Luciferase values were normalized to beta-galactosidase activity from the internal control plasmid BosβGalactosidase (Collingwood et al. 1994) and represent the mean ± SD of 7 independent experiments each performed in triplicate.

Western-blotting

For western blotting, cells were plated and transfected as described above. Forty-eight hours after transfection, they were washed twice with PBS, detached from the plates with PBS-5 mM EDTA/5 mM EGTA (as described by Vilain et al. 2001) and pelleted 5 min at 13,000*g* at 4°C. The pellet was then suspended in 100 µl Laemmli buffer, submitted to three cycles of freeze-thaw, boiled and spun 15 min at 13,000*g* at 4°C. Proteins were quantified on the supernatant with the DC Protein Assay (BioRad). A measure of 15 µg of total proteins were loaded on a 10% SDS-PAGE and electroblotted onto a Hybond ECL membrane (Amersham Bioscience). Membranes were blocked with 5% non fat dry milk in PBS-Tween. Then, the blot was probed overnight at 4°C with a 1:1,000 dilution of anti-FLAG antibody M2 (Sigma-Aldrich), with 1:2,000 dilution of anti-PAX8 antibody (given by Vassart) or with 1:1,000 anti-TTF1 antibody (Dako-Cytomation). After anti-PAX8 antibody, horseradish peroxidase-conjugated swine anti-rabbit antibody (Dako-Cytomation) and after anti-Flag and anti-TTF1 antibodies, horseradish peroxidase-conjugated sheep anti-mouse antibody were used as second antibody. Bound antibodies were revealed with a chemiluminescence Kit (Amersham Bioscience).

Immunocytochemistry

For immunocytochemistry, cells were plated and transfected as described above. Forty-eight hours after transfection, cells were washed, fixed with acetone/methanol (5/95. v/v) for 10 min and permeabilised with PBS-Triton 0.1% for 5 min. Endogeneous peroxidase activity was blocked with 3% H_2O_2 for 10 min. After washing, nonspecific labeling with Universal blocking reagent (Biogenex) was blocked. Cells were then incubated with 1:500 dilution of anti-FLAG antibody M2 or with 1:400 dilution of anti-PHOX2B antibody (given by Lyonnet) for 1 h at room temperature. Controls were performed by omitting the primary antibody. After the anti-flag antibody, Multilink antibody (Biogenex) and after anti-PHOX2B, horseradish peroxidase-conjugated anti-goat were used as secondary antibody. Finally, the immunoreactive products were visualized using the DAB reagent (Biogenex).

Results

Population study

Case–control study

For the case–control study, we sequenced the alanine tracts of the *FOXE1* gene in 115 patients with TD and in 129 French Caucasian controls.

We detected no mutations of the FOXE1 gene other than two already known polymorphisms (L129L and S273S) (Macchia et al. 1999) and polymorphic expansion or contraction of the polyA. Within the polyalanine stretch, we identified six different alleles leading to ten genotypes (Table 2). The alleles encoding 14- and 16-alanine tracts were present in the vast majority of the subjects: there were found in 95.7% of the alleles of patients and in 98.8% of control alleles. Due to the small number of rare alleles (11, 12, 17, 19 alanines identified in only 4.3% of patients and 1.2% of controls) only the 14 and 16 alanine tracts were used. The longer tracts (17 and 19 alanines) and the shorter ones (11 and 12 alanines) were not de novo expansions and were present in one of the unaffected parents of those cases.

The distribution of the 14/14, 14/16, 16/16 and others (corresponding to all the others genotypes) genotypes was

Table 2 Genotypes of patients and controls (according to the length of the polyalanine tract of FOXE1)

Genotypes	Patients								Controls	
	Total		Athyreosis		Ectopy		Hypoplasia/Hemiagenesis			
	Number	Frequency (%)	Number	Frequency (%)	Number	Frequency (%)	Number	Frequency (%)	Number	Frequency(%)
11/14	1	0.9	1	2.9	0	0	0	0	0	0
12/14	1	0.9	1	2.9	0	0	0	0	0	0
12/16	2	1.7	1	2.9	0	0	1	6.3	0	0
14/14	73	63.5	18	51.4	46	71.9	9	56.3	58	45
14/16	28	24.3	10	28.6	15	23.4	3	18.8	51	39.5
16/16	5	4.3	3	8.6	0	0	2	12.5	17	13.2
14/17	3	2.6	0	0	2	3.1	1	6.3	1	0.8
14/19	1	0.9	1	2.9	0	0	0	0	0	0
16/17	0	0	0	0	0	0	0	0	2	1.6
17/19	1	0.9	0	0	1	1.6	0	0	0	0
	115	100	35	100	64	100	16	100	129	100

significantly different in cases and controls ($\chi^2 = 17.2$, *df* 3, $P = 0.0006$; Table 3). The 14/14 genotype was the most frequent both in cases (63.5%) and in controls (45.0%) whereas the 14/16 and 16/16 were less frequent among patients (24.3% in patients vs. 39.5% in controls and 4.3% vs. 13.2%, respectively). As the 14/14 genotype and the 14 allele were the most frequent in the control group, they were used as reference for the OR calculations. Genotypes other than 14/14, 14/16 and 16/16 were not included in the tests.

Statistical analysis revealed an OR for the 16/16 and 16/14 genotypes versus the 14/14 genotype at 0.39 (95%CI = 0.22–0.68, $P = 0.0005$), suggesting that the presence of a 16 alanine tract could protect against the occurrence of TD in comparison to the 14/14 genotype (Table 3). An allele dose effect was even observed, the risk of TD decreased with the number of copies of the allele 16 in the genotype. Indeed, an OR of 0.44 was observed for the 14/16 genotype versus 14/14 (95%CI = 0.24–0.81, $P = 0.004$), whereas an OR of 0.23 was found for the 16/16 genotype versus 14/14 (95%CI = 0.07–0.73, $P = 0.004$) for TD occurrence. These data also signified that the presence of 14/14 genotype was associated with an increase of TD (14/14 genotype *vs.* 14/16 and 16/16; OR = 2.59, 95%CI = 1.56–4.62, $P = 0.0005$).

Furthermore, because the occurrence of TD has a striking sexual dimorphism (two to three affected females for one affected male) (Castanet et al. 2001; Eugene et al. 2005), we conducted the same analysis in males and females separately. Our results remain similar both in males (OR = 0.37 for the 16/16 and 16/14 vs. the 14/14 genotype; 95%CI = 0.14–0.96, $P = 0.024$) and in females (OR = 0.40; 95%CI = 0.19–0.85, $P = 0.009$).

Additionally, since it is uncertain whether athyreosis and ectopy result from the same or from different mechanisms (Gagne et al. 1998), the analysis was performed in the two subgroups separately. We found that patients with ectopy carried allele 16 significantly less often than patients with athyreosis (14.8% vs. 30.0%, $P = 0.016$). Moreover, the proportion of the 16/16 and 16/14 genotypes was significantly lower in the subgroup of patients affected by ectopy than in the control group (23.4% vs. 52.7%, $P = 0.0002$) (Table 4, Fig. 1) while there was no differences between patients affected by athyreosis and the controls (37.2% vs. 52.7 %, $P = 0.2298$). The OR of patients affected by ectopy for the 16/16 and 16/14 genotypes versus the 14/14 genotype was 0.28 (95%CI = 0.13–0.58, $P = 0.00015$). By contrast, in the group of patients affected by athyreosis, this association was not significant (OR = 0.62;

Table 3 Comparison of genotype distribution in patients and controls

Genotypes	Cases		Controls		OR	95%CI	*P*
	N	Frequency	*N*	Frequency			
16/16*	5	4.3	17	13.2	0.23	[0.07–0.73]	0.004
14/16*	28	24.3	51	39.5	0.44	[0.24–0.81]	0.004
14/14	73	63.5	58	45	1		
Others	9	7.8	3	2.3			
Total	115	100	129	100			
*16/16 and 14/16	33	28.7	68	52.7	0.39	[0.22–0.68]	0.0005

Table 4 Odds ratio of genotypes polyalanines FOXE1 in ectopy or athyreosis groups versus controls

Genotypes	Ectopy group		Controls		OR	95%CI	*P*
	N°	Frequency	N°	Frequency			
16/16 and 16/14	15	23.4	68	52.7	0.28	0.13–0.58	0.00015
14/14	46	71.9	58	45.0	1		
Others	3	4.7	3	2.3			
Total	64	100	129	100			
Genotypes	Athyreosis group		Controls		OR	95%CI	*P*
	N°	Frequency	N°	Frequency			
16/16 and 16/14	13	37.2	68	52.7	0.62	0.26–1.46	0.23
14/14	18	51.4	58	45.0	1		
Others	4	11.4	3	2.3			
Total	35	100	129	100			

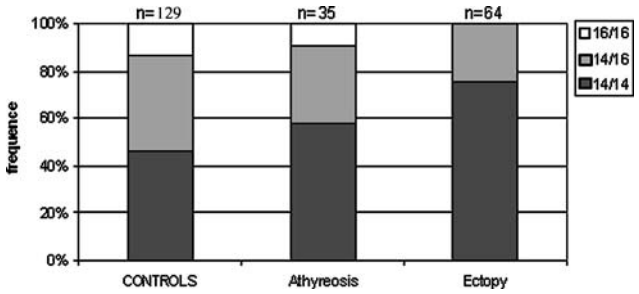

Fig. 1 Frequencies of genotypes (14/14, 14/16 and 16/16) in control group and in two groups of patients, athyreosis and ectopy

95%CI = 0.26–1.46, $P = 0.23$). The association observed in the whole cohort was stronger when only the subgroup of patients affected by ectopy was considered.

Moreover, since monozygotic twins have been shown to be discordant for TD in >90% of the cases (Perry et al. 2002), we also investigated four Caucasian discordant monozygotic twins present in our studied population (i.e., with one having congenital hypothyroidism due to athyreosis or to ectopy and the other being euthyroid). All had the same 14/14 genotype (data not shown).

Transmission disequilibrium test

To confirm the results of the case–control association study, the transmission disequilibrium test (TDT) was used. For this analysis, we considered only parents bearing the 14 and/or 16 alleles with at least one parent being informative (i.e., heterozygous 14/16) which allowed us to select 39 trios. Among them, 26 parents transmitted allele 14 to their affected child while 13 parents transmitted allele 16 ($\chi^2 = 4.3$, $P = 0.0374$).

Moreover, this was even more significant when restricting the TDT analysis to patients with ectopic glands (20 transmitting allele 14 to their affected child while 6 transmitting allele 16; $\chi^2 = 7.5$, $P = 0.006$).

Functional study

As the alleles encoding 14- and 16-alanine tracts were present in the vast majority of patients and controls, we chose to investigate the functional effects of the 14 and 16 residues only by transiently transfecting eukaryotic cells co-expressing the luciferase gene directed by the human thyroglobulin gene promoter. Since in physiological conditions, PAX8, TTF1 and FOXE1 interact, we performed transactivation experiments using these three transctiption factors. As shown in Fig. 2a, luciferase assays revealed that the plasmid containing FOXE1 with 16 alanines induced stronger transactivation than with 14 alanines in the context of synergy with constant amounts of PAX8 and TTF1 (activity over baseline 22.8 ± 3.4 SD vs. 14.7 ± 2 SD, $P < 0.003$) (Fig. 2a). Western blot showed that PAX8, TTF1 and FOXE1 proteins were indeed synthesized in our in vitro experiments (Fig. 2b). Thus, the ability to transactivate a target gene was significantly modified by the length of the alanine tracts.

Furthermore, to investigate whether the cellular localisation of FOXE1 depended upon the length of the alanine stretch, we performed immunocytochemistry (Fig. 3). We detected similar amounts of FOXE1 protein in the nucleus with constructs containing 14 or 16 alanines. In contrast, as previously described (Trochet et al. 2005), we found that the PHOX2B transcription factor aggregated in the cytoplasm when containing 33 alanines instead of 20 alanines. These PHOX2B aggregates sometimes spread over the whole cytoplasm or concentrated near the nucleus (Trochet et al. 2005). This experiment showed that misfolding of FOXE1 with 14 or 16 alanines is not the mechanism that explained the differences in the transactivation abilities.

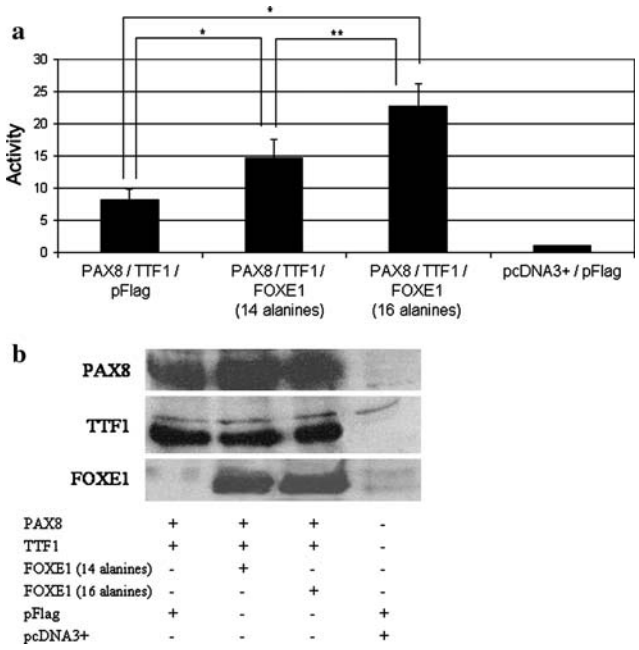

Fig. 2 Transfection of FOXE1 in 293A cells: **a** effects of 14 or 16 alanines on transactivation. Effects of the alanine tract of FOXE1 (with 14 or 16 alanines) coexpressed with PAX8 and TTF1 on transcriptional activity. The data represent the mean ± SD in 7 assays. $^{**}P < 0.003$ (Mann–Whitney test). $^{*}P < 0.002$ (Mann–Whitney test). **b** effects of 14 or 16 alanines on protein expression. Expression of FOXE1, PAX8 and TTF1 proteins in 293A cells assessed by western blot. Each of these proteins was expressed in 293A cells. pFlag and pcDNA3+ were the empty vectors

Discussion

Alanine-tract expansions in transcription factors have been implicated as a cause of some human diseases. One example is congenital central hypoventilation syndrome (OMIM 209880) where expansions of the alanine tract in *PHOX2B*, a paired-type homeobox transcription factor, have been directly linked to the disease (Amiel et al. 2003).

FOXE1 is a transcription factor implicated in thyroid development. There is strong evidence for FOXE1 involvement in thyroid development as some human mutations have been reported. However, the implication of its polyA tract in TD remains so far unclear. Recently, three groups found polymorphism of the polyA stretch lengths varying from 11 to 16 residues with the 14 as the most frequent allele both in TD patients and controls (Hishinuma et al. 2001; Tonacchera et al. 2004; Santarpia et al. 2007). Furthermore, another group performed polyA analysis in FOXE1 in premature ovarian failure showing stretch length varying from 12 to 19 residues as in our study (Watkins et al. 2006). In our study, we investigated a larger cohort than previously studied, which allowed us to perform not only a case–control association study but also TDT analysis. Our results strongly suggest that the length of the alanine stretch within FOXE1 modulates genetic susceptibility to TD. Indeed, patients affected by TD present a significantly lower proportion of the 16/16 and 16/14 genotypes compared to controls (28.7% vs. 52.7%). The OR at 0.39 strongly suggests that the presence of 16 alanines either at heterozygote state (i.e., 14/16 genotype) or at homozygote state (i.e., 16/16 genotype) significantly protects from the occurrence of TD in comparison with the 14/14 genotype. Thus, TD is associated with the more common variant (allele 14) suggesting that the less common variant (allele 16) may offer protection against developing the disease (Sladek et al. 2007; Freimer and Sabatti 2007). In our work, alternatively one can consider the presence of the 14/14 genotype as associated with an increase risk of TD. It may be surprising that the most common genotype (i.e., 14/14 genotype) in our cohort of controls as well as in affected cases confers risk but this has been previously described for some other gene polymorphisms and diseases (Malik et al.

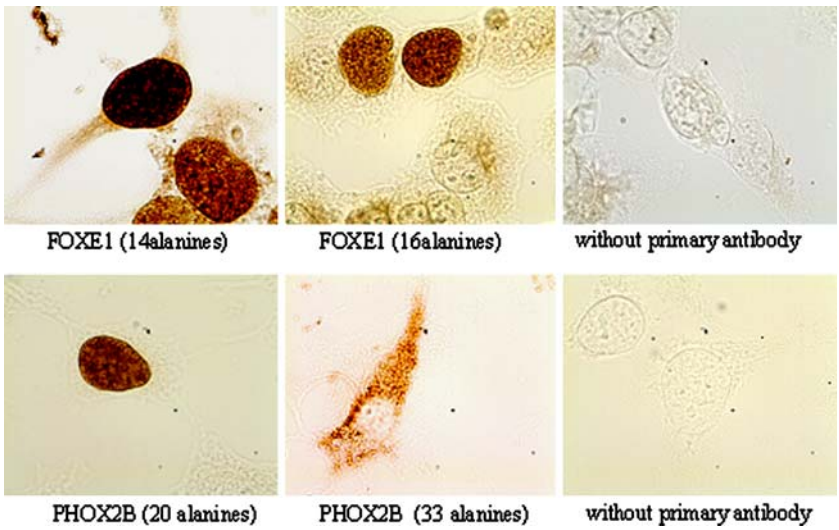

Fig. 3 Localisation of transcription factors FOXE1 and PHOX2B in 293A cells. The cells were examined by immunocytochemistry. The length of the polyalanine tract influences the localisation of PHOX2B but not of FOXE1 (×1000)

2005). Additionally, the fact that protection against the occurrence of TD was more significant for ectopy might be in accordance with the role of Foxe1 in thyroid development. Indeed, knockout mice data suggested that Foxe1 could control migration of the follicular cells from the foramen caecum of the tongue to the neck and their terminal differentiation (De Felice and Di Lauro 2004).

Note that although all of the four previously reported studies of the polyA found that allele 14 is the most frequent in the control group (Hishinuma et al. 2001; Tonacchera et al. 2004; Watkins et al. 2006; Santarpia et al. 2007) alleles distribution is different (Table 1). In our study, the allelic distribution of control group differ significantly from those described by the two Italian and the Japanese groups (Hishinuma et al. 2001; Tonacchera et al. 2004; Santarpia et al. 2007) but is consistent with those reported by another Italian group (Macchia et al. 1999) and New Zealand and Slovenian groups (Watkins et al. 2006). These difference might reflect ethnicity-related deviations and could explain the difference between our result and the recently described by Santarpia et al. (2007). Indeed in this study, the homozygous allele 14 was less frequent in the patient group than in the control group. None of these groups performed the most rigorous analysis that is TDT study in addition to a case–control association study (Hishinuma et al. 2001; Tonacchera et al. 2004; Santarpia et al. 2007). Nevertheless, the association observed with the alanine tract does not exclude the possibility that the polymorphism in question is itself neutral and that the biologically relevant variant is in linkage disequilibrium with the one being studied. However, the fact that the same association has been reported in a different population adds credence to the idea that the alanine tract itself is the relevant polymorphism, as does also the functional study (see below).

Thus, our analysis allowed us to conclude that FOXE1 through its alanine containing stretch modulates significantly the risk of TD occurrence. This effect was even more marked in the group of patients with an ectopic gland. By contrast, in the group of patients affected by athyreosis, this association was not significant.

Although the statistical analysis demonstrates a strong association between thyroid dysgenesis and variation of polyA length, it provides no evidence for a direct role in the abnormal thyroid development. Indeed, many of the controls have the 14/14 genotype and yet do not have TD, suggesting that the length of the alanine tract within *FOXE1* is not exclusively responsible for abnormal thyroid development. The finding in the monozygotic twins also suggests that the genetic susceptibility of TD is affected by factors other than the length of the FOXE1 polyA tract.

Taken together, our study is distinct from the others in so far that the alanine-tract expansion of FOXE1 is a susceptibility factor and not a disease-causing mutation (Amiel et al. 2003; Abu-Baker and Rouleau 2007).

Based on our systematic statistical analysis, we aimed to test the effect of polyA tract variation on protein function. We performed transfections studies with longer polyA tract lengths (14 vs. 16 alanines) and demonstrated for the first time that the length of the alanine tract within FOXE1 modified the transactivation ability in the presence of TTF1 and PAX8, a combination of transcription factors whose simultaneous expression is unique to thyroid follicular cells (Sura-Trueba et al. 2005). Using such combination, we were able to show differences between transcriptional activities of polymorphisms demonstrating the biological relevance of variation in the length of FOXE1 polyA tract (14 vs. 16 alanines).

Furthermore, expansions of polyA tracts result in protein misfolding and aggregation for some transcriptions factors (Albrecht and Mundlos 2005, Shoubridge et al. 2007). Our in vitro assays with alterations in alanine-tract length illustrate that the nuclear localisation of FOXE1 was not modified by the expanded alanine tract (14 vs. 16) in contrast to what has been showed for PHOX2B gene (20 vs. 33). Additionally, no aggregate formation was visible in either cytoplasm or the nucleus. Thus, to explain variations of transcriptional activity, we suggest a modified conformation linked to the number of alanines that may change the interaction of the transcription factor complexes with target genes, as previously postulated (Lavoie et al. 2003).

In summary, our data strongly point to a role of the FOXE1 polyA tract length in TD, enhancing an action of an alanine containing transcription factor in relation to disease. Nevertheless, multifactorial origin of TD is now recognised (Amendola et al. 2005) and variations of polyA tract could be considered as a modulator of genetic susceptibility for TD in such a polygenic model.

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