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# “Thyroid Dysgenesis: search for mutations and functional characterizations of known and new candidate genes”

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# “Thyroid Dysgenesis: search for mutations and functional characterizations of known and new candidate genes”

*To my family..*

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# LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Selenium in the thyroid: physiology and pathology
   Carolina De Fusco, **Immacolata Cristina Nettore**, Annamaria Colao, Paolo Emidio Macchia. *Reviews in Endocrinology and Metabolism 2013; 1 (1): 34-40*1.

2. The molecular causes of thyroid dysgenesis: a systematic review
   **Nettore IC**, Cacace V, De Fusco C, Colao A, Macchia PE. *J. Endocrinol. Invest.2013 Sep; 36: 654-664.*

3. Identification and functional characterization of a novel mutation in the NKX2-1 gene: comparison with the data in the literature
   **IC Nettore**, P Mirra, AM Ferrara, A Sibilio, V Pagliara, CS Kamoi Kay, PJ Lorenzoni, LC Werneck, I Bruck, LH Coutinho dos Santos, F Beguinot, D Salvatore, P Ungaro, G Fenzi, RH Scola & PE Macchia. *Thyroid. 2013 Jun; 23(6):675-82*

4. Screening for mutations in the isl1 gene in patients with thyroid dysgenesis.
   Ferrara AM, Rossi G, Zampella E, Di Candia S, Pagliara V, **Nettore IC**, Capalbo D, De Sanctis L, Baserga M, Salerno MC, Fenzi G, Macchia PE. *J Endocrinol Invest. 2011 Jul-Aug;34(7):e149-52*

# LIST OF ABBREVIATIONS

| | |
|---|---|
| BHC | Benign hereditary chorea |
| BTLS | brain-thyroid-lung syndrome |
| CH | congenital hypothyroidism |
| HRM | high resolution melting |
| LUC | luciferase |
| MUT | mutant |
| NKX2-1 | thyroid transcription factor 1 |
| NKX2-5 | thyroid transcription factor 5 |
| PAX8 | paired box 8 |
| PCR | polymerase chain reaction |
| rTSH | thyroid stimulating hormone receptor |
| SNPs | single nucleotide polimorphisms |
| SP-C | surfactant protein C |
| SSCP | single strand conformism polymorphism |
| TD | thyroid dysgenesis |
| TFC | thyroid follicular cell |
| Tg | thyroglobulin |
| TPO | thyroperoxidase |
| WT | wild-type |

# ABSTRACT

Background: Alterations in thyroid organogenesis occurs very frequently in humans, leading to several conditions responsible for primary congenital hypothyroidism. Such alterations, classified as "thyroid dysgenesis" (TD) include athyresis, ectopy of the gland, and thyroid hypoplasia. The molecular mechanism leading to TD are largely unknown, but, several studies linked thyroid dysgenesis to mutations in genes expressed in the developing thyroid, such as *NKX2-1, NKX2-5, PAX8, FOXE1, rTSH*.
*NKX2-1* mutations have been described in several patients with a syndromic form of primary congenital hypothyroidism associated to respiratory distress, and benign hereditary chorea (Brain–Thyroid–Lung Syndrome, BTLS).
Methods: we screened 76 patients with persistent CH for mutations in known (*NKX2-1, NKX2-5, PAX8)* and new candidate (*DNAJC17*) genes by SSCP, or HRM. In addition, the *NKX2-1* gene was sequenced in the members of a Brazilian family with clinical features of BTLS.
Results: We found no mutations in the *PAX8* or *NKX2-5* genes in our cohort. The only variation observed was an already reported polymorphism (rs199939219) within the *PAX8* gene that causes a change of the first nucleotide of the triplet encoding for serine at position 337 (Ser337Ala). This change, functionally tested by *in vitro* studies, has no effect on the PAX8 protein activity.
We also looked for mutations in the *DNAJC17* gene, a new candidate gene that has been demonstrated to act as phenotype modulator in mouse double heterozygous for deletion in *Nkx2-1* and *Pax8*. In *DNAJC17* we found a known polymorphism c.350A>C.
Finally, we were able to identify a novel *NKX2-1* mutation in the Brazilian family with the clinical findings of the BTLS. The mutation is a deletion of a cytosine at position 834 (ref. sequence NM_003317) (c.493delC) that causes a frameshift with formation of an abnormal protein from amino acid 165 and a premature stop at position 196. The last amino acid of the nuclear localization signal, the whole homeodomain, and the carboxy terminus of NKX2-1 are all missing in the mutant protein, which has a premature stop codon at position 196 (p.Arg165Glyfs*32). The p.Arg165Glyfs*32 mutant does not bind DNA, and it is unable to transactivate the thyroglobulin (Tg) and the surfactant protein-C (SP-C) promoters. Interestingly, a dose-dependent dominant negative effect of the p.Arg165Glyfs*32 was demonstrated only on the Tg promoter, but not on the SP-C promoter. This effect was also noticed when the mutation was tested in presence of PAX8 or cofactors that synergize with NKX2-1 (P300 and TAZ). The functional effect was also compared with the data present in the literature and demonstrated that, so far, it is very difficult to establish a specific correlation among NKX2-1, PAX8 and NKX2-5 mutations, their functional

consequence, and the clinical phenotype of affected patients, thus suggesting that the detailed mechanisms of transcriptional regulation still remain unclear.
Conclusions: We describe a novel NKX2-1 mutation in a Brazilian family and demonstrate that haploinsufficiency may not be the only explanation for BTLS. Our results indicate that NKX2-1 activity is also finely regulated in a tissue-specific manner, and additional studies are required to better understand the complexities of genotype–phenotype correlations in the NKX2-1 deficiency syndrome. No mutations have been identified in sporadic cases of TD in *NKX2-5, PAX8 or DNAJC17* genes, suggesting that sporadic cases of TD are likely to be caused by epigenetic factors, rather than mutations in thyroid transcription factors or genes involved in thyroid development.

# 1. BACKGROUND

Primary congenital hypothyroidism (CH) is the most frequent endocrine metabolic disease in the infancy, with an incidence of about 1/2500 newborns a year (Cassio, Corbetta et al. 2013). In absence of an adequate treatment, CH is characterized by signs and symptoms of impaired metabolism and by motor and mental handicaps. Before the introduction of a neonatal screening program, CH was one of the most frequent causes of mental retardation. With the exception of the rare cases of central hypothyroidism, CH is characterized by the presence of elevated TSH levels at birth in response to reduced thyroid hormone production.

Many human disorders appear to be derived from alterations of the morphogenetic events. For instance, severe malformations in foregut-derived organs (such as esophageal atresia, trachea esophageal fistula, lung anomalies, and congenital stenosis of the esophagus and trachea) are common anomalies occurring in one in 2000 to one in 5000 live births (Motoyama J, Liu J et al. 1998).

Thyroid gland organogenesis results in an organ the shape, size, and position of which are largely conserved among adult individuals of the same species, thus suggesting that genetic factors must be involved in controlling these parameters.

In humans, the organogenesis of the thyroid gland is often disturbed, leading to a variety of conditions. In the majority of cases (80-85%), CH is due to alterations occurring during the gland organogenesis, which results in a thyroid gland that is absent (thyroid agenesis or athyreosis), hypoplastic (thyroid hypoplasia), or located in an unusual position (thyroid ectopy). All these entities are grouped under the term “thyroid dysgenesis” (TD) (De Felice M and Di Lauro R 2004).

In the remaining 15-20% of cases, CH is caused by inborn errors in the molecular steps of thyroid hormones biosynthesis, secretion or recycling. These forms of CH are indicated with the term “dyshormonogenesis”, and are characterized by an enlargement of the gland (goiter), presumably due to elevated TSH levels. Thyroid dyshormonogenesis shows classical Mendelian recessive inheritance, and the molecular mechanisms in most of these forms have been characterized. In addition, mostly thanks to the changes in screening strategies for CH of the last years, it has been recently observed an increase number of mild forms of CH associated with eutopic thyroid gland. Of these, about one third of patients presented a permanent hypothyroidism and need to continue LT4 therapy after re-evaluation, and in 8.7% of the studied cases a mutation in TSHR gene has been identified (Rabbiosi, Vigone et al. 2013).

*1.1 Morphogenesis of thyroid gland*

The thyroid gland in mammals is located in the neck region. The gland produces thyroid hormones and calcitonin in two distinct cell types, the TFCs and the parafollicular or C cells, respectively. The TFCs, the most numerous cell population in the gland, form the thyroid follicles, spherical structures serving as storage and controlled release of thyroid hormones (Mauchamp J, Mirrione A et al. 1998). The C cells are scattered in the interfollicular space, mostly in a parafollicular position. The two diverse cell types, responsible for the dual endocrine function of the gland, originate from two different embryological structures: the thyroid anlage is the site of origin of the TFCs whereas the ultimobranchial bodies are the source of C cells. The thyroid anlage is an area enclosing a small group of endodermal cells, and it is located on the midline of the embryonic mouth cavity in its posterior part. The ultimobranchial bodies are a pair of transient embryonic structures derived from the fourth pharyngeal pouch and located symmetrically on the sides of the developing neck. The C cell precursors migrate from the neural crest (Le Douarin N, Fontaine J et al. 1974) bilaterally to the fourth pharyngeal pouches and become localized in the ultimobranchial bodies (Fontaine J 1979). The cells of the thyroid anlage and the ultimobranchial bodies migrate from their respective sites of origin and ultimately merge in the definitive thyroid gland. In the merging process, both the thyroid anlage and the ultimobranchial bodies disappear as individual structures, and the cells contained in them disperse in the structure of the adult thyroid gland. The cells originating from the anlage continue to organize the thyroid follicles, whereas the C cells scatter within the interfollicular space.
The morphogenesis of the thyroid, like in many endoderm-derived organs, begins with an event that recruits a group of cells, in an homogeneous cell layer, to the thyroid fate, i.e., to follow all the developmental steps necessary to achieve the TFC phenotype. This event is called, in developmental biology terms, "specification", because a group of cells are specified to undertake a definite developmental program. The first visible change is a thickening of the endodermal epithelium in the foregut, which is referred to as thyroid anlage. Thickening in a restricted region of a cell layer, a common event in the initiation of organogenesis, has been suggested to be an essential event in the generation of signals required for the continuation of organogenesis (Kenyon KL, Ranade SS et al. 2003).

In mice, thyroid anlage appears at embryonic day (E) 8-8.5 in the ventral wall of the primitive pharynx (Kaufman MH and Bard J 1999). At E9-E9.5 the endodermal cells of the thyroid anlage start to migrate and to proliferate, invading the surrounding mesenchyme and forming the thyroid bud. By E10, the thyroid primordium appears as a flask-like structure with a narrow neck that rapidly becomes a diverticulum. A small hole at the site of origin in the pharyngeal floor (the foramen cecum) is the remnant of the anlage, connected

with the migrating thyroid primordium by a narrow channel (the thyroglossal duct). At E11.5 the thyroglossal duct disappears, and the thyroid primordium loses its connections with the floor of the pharynx and begins to expand laterally. Two days later the thyroid primordium reaches the trachea, which has extended ventrocaudally starting from the primitive laryngotracheal groove. By E15–E16 the thyroid lobes expand considerably, and the gland exhibits its definitive shape: two lobes connected by a narrow isthmus. The mechanisms leading to proliferation of the precursors and to the formation of the lobes remain to be elucidated. Surprisingly, TSH signaling, the best known growth stimulus for adult thyroid cells, does not appear to be involved (Postiglione MP, Parlato R et al. 2002) (Marians, Ng et al. 2002). By E15.5, the first evidence of follicular organization appears with many small follicles disseminated within the gland. At this time calcitonin-producing C cells, derived from the ultimobranchial bodies that have fused with the primitive thyroid at around E14, can also be detected among follicles (Cordier AC and Haumont 1980).
The differentiative program of TFCs is completed only when the gland reaches its final location. As a result, TFCs express a series of proteins that are typical of TFCs and that are essential for thyroid hormone biosynthesis. Because there will be no further differentiation event in the life of TFCs, this last differentiation could be called terminal or functional differentiation. Genes typical of this stage appear according to a given temporal pattern: Tg, TPO, and TSH receptor (Tshr) genes are expressed by E14.5 (Lazzaro, Price et al. 1991) (35); sodium/iodide symporter (NIS) is detected by E16 (Postiglione MP, Parlato R et al. 2002). T4 is first detected at E16.5 (Meunier D, Aubin J et al. 2003).

In humans, the process of thyroid morphogenesis is very similar, lasting from the 3rd to 16th week of gestation (Trueba SS, Auge J et al. 2005).

## *1.2 Thyroid dysgenesis*

The most critical events in thyroid organogenesis occur during the first 60 days of gestation in man and the first 15 days in mice. It is likely that alterations in the molecular events occurring during this period can be associated to TD.

### 1.2.1 Athyreosis

The absence of TFC in orthotopic or ectopic location is called *athyreosis*. This condition can either be the consequence of lack of formation of the thyroid bud or results from alterations in any of the step following the specification of the thyroid bud and determining a defective survival and/or proliferation of the

precursors of the TFC. In athyreotic patients, the presence of cystic structures resulting from the persistence of remnants of the thyroglossal duct is frequently reported (Marinovic D, Garel C et al. 2003). This finding indicates that in these subjects some of the early events of thyroid morphogenesis have taken place but the cells fated to form the TFCs either did not survive or switched to a different fate. In many cases, scintigraphy failed to demonstrate the presence of thyroid tissue, but thyroid scanning by ultrasound reveals a very hypoplastic thyroid.

### 1.2.2 Ectopia

The *ectopic thyroid* is the consequence of a failure in the descent of the developing thyroid from the thyroid anlage to its definitive location in front of the trachea. An ectopic thyroid can be found in any location along the path of the developing gland migration from the foramen caecum to the mediastinum. In the majority of cases, the ectopic thyroid appears as a mass in the back of the tongue (lingual thyroid, usually functioning). Sublingual ectopic tissues are less frequent; in this case, thyroid tissue is present in a midline position above, below or at the level of the hyoid bone. Ectopic thyroid tissues within the trachea have also been reported (Brandwein M, Som P et al. 1998). In a few cases thyroid tissue in the submandibular region has been described (Kumar R, Gupta R et al. 2000) (Feller KU, Mavros A et al. 2000). Some authors, on the assumption that TFCs derive from both a median thyroid and a lateral thyroid bud (*i.e.*, the ultimobranchial body), hypothesize that this aberrant thyroid tissue originates from a defective lateral thyroid component that cannot migrate and fuse with the median thyroid anlage. However, the hypothesis that TFCs can be derived from the ultimobranchial bodies appears to be questionable. Studies in animal models did not offer any conclusive demonstration that cells of ultimobranchial bodies are fated to differentiate toward typical TFCs. Furthermore, in the majority of subjects with an ectopic thyroid located along the midline, no other thyroid tissue is detectable either in paratracheal or in tracheal areas. If the thyroid did originate from two different buds, a higher number of cases where, in addition to the ectopic median thyroid, another thyroid mass derived from the lateral component should also be present. Because this finding has never been reported, we should conclude that ectopic submandibular thyroid tissues, also, are the result of an aberrant migration of the median thyroid anlage (De Felice M and Di Lauro R 2004). Developmental defects other than abnormal migration of the thyroid bud should be taken into account to explain the presence of thyroid tissue in locations distant from the path of migration of the embryonic gland. The origin of intracardiac ectopic thyroids could be due to disturbances occurring early in embryogenesis, when the thyroid anlage is in close contact with the embryonic heart (Casanova JB, Daly RC et al. 2000). In the case of sub diaphragmatic locations such as the duodenum wall (Takahashi T, Ishikura H et al. 1991),

gallbladder (Harach HR 1998), or porta hepatis, either aberrant migration or heterotopic differentiation of uncommitted endodermal cells could be hypothesized (Ghanem N, Bley T et al. 2003).

### 1.2.3 Hypoplasia

Orthotopic and *hypoplastic thyroid* is reported in 5% of CH cases. Thyroid hypoplasia is probably a genetically heterogeneous form of thyroid dysgenesis. It could be due to defects in any of the genes that control the number of thyroid cells (De Felice M and Di Lauro R 2004).
The relative proportions of the main TD phenotypes vary in different studies, depending on the methodology used to detect the presence of the gland. According to $^{99}$Tc scintigraphy (Olivieri, Stazi et al. 2002), (Devos, Rodd et al. 1999), (Connelly JF, Coakley JC et al. 2001), more sensitive but exclusively dependent on metabolic activity, ectopy of the thyroid is the most frequent type of dysgenesis (48–61% of cases), whereas athyreosis is the cause of 15–33% of cases of TD. The scintigraphic analysis could either not reveal the frequency of thyroid hypoplasia, or most likely underestimated it at only 5% of CH patients. It is conceivable that these studies included in athyreosis some non functioning thyroids and counted as hypoplasia only the smallest glands. In a study based on ultrasound diagnosis, which is independent from metabolic activity but not as sensitive as scintigraphy, athyreosis was found in 48% of CH patients, ectopy in 18%, and hypoplasia in 17%. This technique most likely included in athyreosis some very small, ectopic thyroids. From these data it could conclude that the most frequent form of TD is thyroid gland ectopy and that a combination of ultrasound sonography and $^{99}$Tc scintigraphy might resolve a precise relative proportion of these phenotypes. However, cost-benefit considerations should be carefully evaluated before performing $^{99}$Tc scintigraphy in cases of CH (De Felice M and Di Lauro R 2004).

### 1.2.4 Hemiagenesis

Thyroid *hemiagenesis* is a rare congenital abnormality, in which one of the thyroidal lobes fails to develop. Thyroid hemiagenesis is often associated with mild and or transient hypothyroidism but several patients were found to be in euthyroid state (both serum TSH and thyroid hormone levels are within the normal range). Thyroidal hemiagenesis is predominantly seen in females with an incidence to 0.2% in healthy children. In the large majority of the cases, it is the left lobe to be absent (Maiorana, Carta et al. 2003). The causes of thyroid hemiagenesis are still unclear and it is unknown whether the disturbance of the lobulation process is due to interference of environmental factors or to genetic abnormality. In addition, the occurrence of some cases of thyroid hemiagenesis among members of the same family (Rajmil HO, Rodriguez-Espinosa J et al. 1984) suggests that genetic factors could be involved in this anomaly. The

molecular mechanisms leading to the formation of the two thyroid symmetrical lobes, which are impaired in the case of hemiagenesis, are not known. In the mouse embryo, by E12, the midline-located thyroid bud begins to expand laterally, and at E15 the bilobed shape of the gland is evident. The genetic basis of the lobulation process is finally beginning to be understood. Indeed, either a non lobulated gland or hemiagenesis of the thyroid (Fagman H, Grände M et al. 2003) have been described in *Shh*$^{-/-}$ mice embryos. Hemiagenesis has also been reported in double heterozygous *Titf1*$^{-/-}$ *Pax8*$^{-/-}$ mice (De Felice, Macchia PE et al. 2002). However, in humans, candidate genes responsible for the hemiagenesis of the thyroid have not yet been described.

### *1.3 Molecular basis of thyroid organogenesis*

The molecular basis of thyroid gland development began to be investigated with the discovery that the transcription factor Nkx2-1, identified as responsible for the thyroid-specific expression of Tg and TPO, is expressed not only in functioning thyroid cells but also in their precursors (Lazzaro, Price et al. 1991). Subsequently, the transcription factors Foxe1, Pax8, and Hhex were also found to be expressed both in mature thyroid cells and in their precursors. The expression of these factors in the thyroid anlage, at the very beginning of thyroid morphogenesis, immediately suggested that these genes might play an important role in the organogenesis of the thyroid gland. These factors are also present in other embryonic tissues, but all four are coexpressed only in the thyroid anlage.

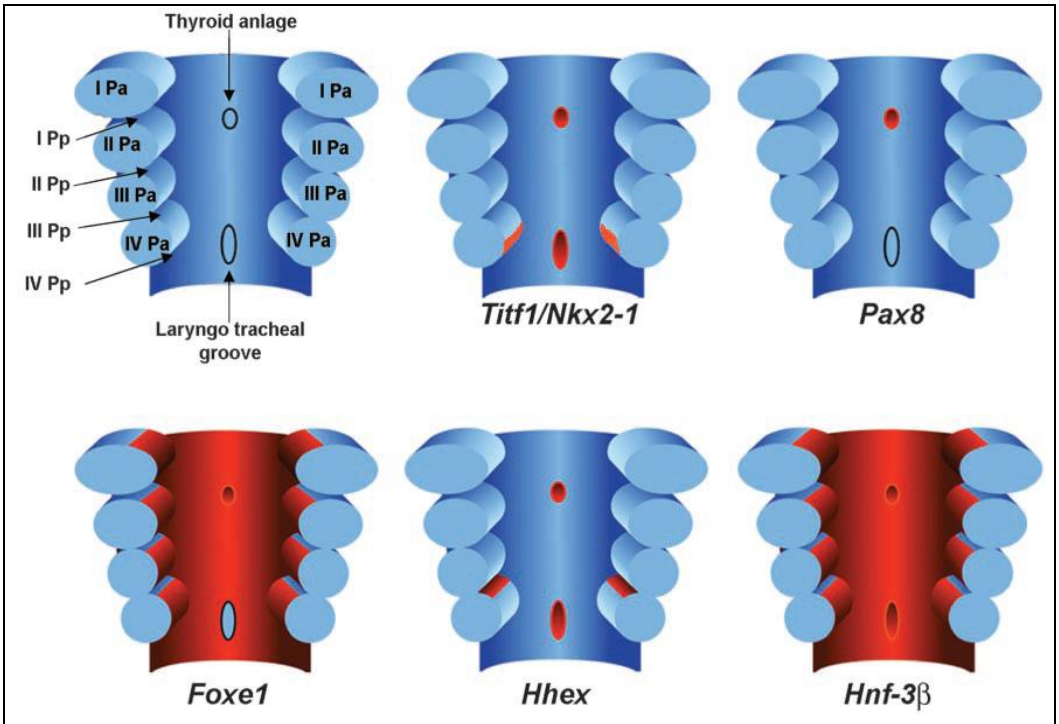
In the primitive pharynx, Nkx2-1 is present exclusively in the thyroid anlage, and its appearance coincides with specification of the anlage. Nkx2-1 remains expressed in the TFC during all stages of development and in adulthood. In differentiated TFC, Nkx2-1 controls thyroglobulin and thyroperoxidase expression (Civitareale, Lonigro et al. 1989). Nkx2-1 is also present in the trachea and lung epithelium **(**Figs. 1 and 2**)** and in selected areas of the forebrain, including the developing posterior pituitary (Lazzaro, Price et al. 1991). After birth and in adult organisms, Nkx2-1 is still present in the thyroid and lung epithelium and in the posterior pituitary (Guazzi, Price et al. 1990), whereas its expression in the brain is restricted to the periventricular regions and some hypothalamic nuclei (Nakamura K, Kimura S et al. 2001). The expression of Nkx2-*1* in the hypothalamus is reduced in the adult compared with the embryonic brain; however, it increases transiently but markedly before the first endocrine manifestations of puberty (Lee BJ, Cho GJ et al. 2001).

Like Nkx2-1, Pax8 is detected in the developing thyroid from E8.5, *i.e.*, at the time of specification. In the endoderm *Pax8* mRNA is present only in the thyroid anlage (Fig. 1). Expression of *Pax8* is maintained in TFCs during all stages of development **(**Fig. 2**)** and in adulthood.

*Foxe1* mRNA is detected at E8.5 in all the endodermal cells of the floor of the foregut, including the thyroid anlage. Hence, at variance with *Nkx2-1* and

*Pax8*, the expression of which in the pharynx is strictly limited to the thyroid anlage, *Foxe1* has a wider domain of expression. However, the expression of *Foxe*1 is limited posteriorly because no *Foxe1* mRNA is present in the lung **(**Fig. 1**)**. It has also been noted that at E8.5 *Foxe1* seems to be much more evident in a region of the pharynx posterior to that of the thyroid precursor cells (Zannini, Avantaggiato et al. 1997). Expression of *Foxe1* in the thyroid cell precursors is maintained during development **(**Fig. 2**)** and persists in adult TFCs. At later stages of development *Foxe1* is expressed in the tissues derived from the pharyngeal arches and pharyngeal wall: thyroid, tongue, epiglottis, palate, and esophagus.

Also *Nkx2-5* is expressed in the thyroid primordium during development (Kasahara, Bartunkova et al. 1998). NKX2-*5* transcripts were identified in the pharyngeal endoderm at stage E8.5 to E9.5 in a subset of cells that start to differentiate and migrate antero-ventrally to constitute the thyroid gland. At later stages, pharyngeal expression of *NKX2-5* is limited to the area corresponding to the thyroid primordium (Chen and Schwartz 1995) and is undetectable thereafter.




De Felice M and Di Lauro R. Endocr ine Reviews 2004

**Fig. 1**. Gene expression in the thyroid region at the beginning of organogenesis. The expression domains on relevant genes are indicated in *red* in a schematic frontal view of the entire pharynx of an E9 mouse embryo. Pp, Pharyngeal pouch; Pa, pharyngeal arch.

Thus, the small number of cells in the primitive pharynx fated to become TFCs already at E8.5 are univocally characterized by the simultaneous expression of Nkx2-1, (Lazzaro, Price et al. 1991) Foxe1 (Zannini, Avantaggiato et al. 1997), Pax8 (Plachov D, Chowdhury K et al. 1990), and Hhex (Thomas PQ, Brown A et al. 1998). When the thyroid diverticulum forms and begins its migration, the expression of these factors is restricted to the thyroid primordium as they are never expressed in the thyroglossal duct. For the rest of its life, a thyroid cell will be hallmarked by the simultaneous presence of Nkx2-1, Foxe1, Pax8, and Hhex (Fig. 1). The expression of these four factors is required for the early stages of thyroid morphogenesis. Therefore, alterations in any of these genes can be responsible for an abnormal thyroid gland morphogenesis and thyroid dysgenesis.

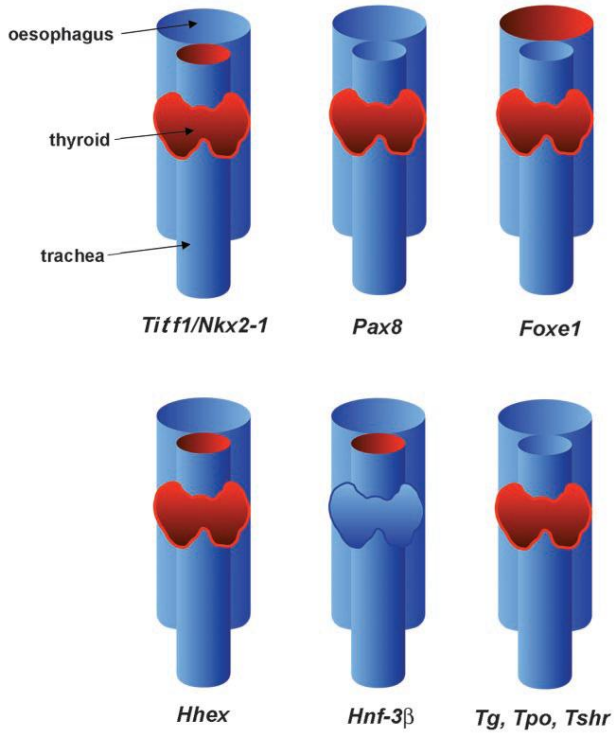



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**Fig. 2**. Gene expression in the thyroid region after completion of organogenesis. The expression domains on relevant genes are indicated in *red* in a schematic frontal view of the trachea esophagus region of an E17 mouse embryo.

*1.4 Genes involved in thyroid dysgenesis*

Because familial occurrence of TD is rare, this condition was considered a sporadic disease resulting mainly from nongenetic causes such as environmental factors or stochastic events during embryogenesis. But, a genetic cause of the disease has been demonstrated in about 5% of the reported cases. TD presents a clear female prevalence (Castanet, Polak et al. 2001). One report (Devos, Rodd et al. 1999) suggested that the female prevalence is significant for ectopy but not for athyreosis. Epidemiological studies demonstrated a different incidence of the disease in different ethnic groups (Lorey FW and Cunningham GC 1992), (Castanet, Polak et al. 2001) suggesting that the genetic background plays a role in this affection. Furthermore, in populations where consanguineous marriages are common, the incidence of CH is increased (Ordookhani A, Mirmiran P et al. 2003). Other evidence in favor of the relevance of genetic factors in CH with TD is the finding of a small but significant proportion of familial cases. It has been reported that 2% of patients had an affected relative (Castanet, Polak et al. 2001). This frequency is 15-fold higher than the frequency expected on the basis of chance alone, indicating that the involvement of genes required for correct thyroid morphogenesis is very likely in these familial forms. Interestingly, in some familial cases the affected members show either athyreosis or ectopy. This finding supports the hypothesis that athyreosis and thyroid ectopy could have common underlying mechanisms, as strongly suggested by the observation that mice deprived of *Foxe1* gene products show either ectopy with a very small thyroid or no thyroid at all (De Felice M, Ovitt C et al. 1998). In addition, among the first-degree relatives of patients with sporadic TD, there is a significantly higher rate of asymptomatic thyroid developmental anomalies compared to the normal populations. Indeed, the prevalence of these anomalies is less than 1% in the control population, whereas it is 8% in first-degree relatives of patients with CH. This suggests the hypothesis that both severe forms of TD and heterogeneous asymptomatic alterations (including hemiagenesis or ectopy of the thyroid, thyroglossal duct cysts, pyramidal lobe) could originate from the same genetic defects during thyroid morphogenesis. Interestingly, in asymptomatic anomalies there is no female preponderance (Leger J, Marinovic D et al. 2002). By contrast, against the notion of heritable TD is the finding reported by Perry et al., showing a discordance for TD in 12 of 13 monozygotic twin pairs (Perry, Heinrichs et al. 2002). This finding suggests that postzygotic events must be evoked in the pathogenesis of many cases of TD. However, it is most likely that the targets of such events are the same genes responsible for normal thyroid development. De Felice and Di Lauro, suggest that the function of genes involved in normal thyroid development can be interfered with by either mutations or epigenetic events. In both cases the result is TD, which would be inheritable, of course, only in the case of genetic mutations. According to different reports (Olivieri, Stazi et al. 2002), (Roberts HE, Moore CA et al. 1997), (Devos, Rodd et al.

1999), (Lazarus and Hughes 1988), TD is associated with other major birth defects in 5–16% of cases. It is conceivable that, in the cases presenting multiple congenital anomalies, the defective mechanism or gene could be involved in the morphogenesis of the thyroid and of the other embryonic structures altered. In this respect, it is of great interest that the malformations most frequently associated with TD are cardiac malformations. The preferential association of thyroid malformations with those of the heart is consistent with the hypothesis that signals generated in the heart primordium are necessary for normal thyroid development.
It should be stressed, however, that studies on thyroid development in normal and mutated mouse embryos indicate the simultaneous presence of *Pax8*, *Nkx2-1*, *Foxe1*, and *Hhex* and that these factors are required for thyroid morphogenesis. Indeed, thyroid dysgenesis is present in animal models with mutations in these genes, and mutations in the same genes have been identified in patients with congenital hypothyroidism associated with TD.

### 1.4.1 *NKX2-1*

NKX2-1 (also known as NKX2-1) is a transcription factor containing a homeodomain. It belongs to the NK2 class of homeobox proteins that have a tyrosine residue at amino acid 54 of the homeodomain and a conserved 23-amino-acid NK2-specific domain (Chen and Schwartz 1995). The human gene maps to chromosome 14q13 and it is formed by 3 exons. NKX2-1 contains three functional domains: an N-terminal transactivation domain, a DNA-binding domain (HD), and a C-terminal transactivation domain (De Felice M, Damante G et al. 1995). There is compelling evidence that NKX2-1 functions by forming complexes with other transcription factors on the regulatory regions of target genes. NKX2-1 interacts with retinoic acid receptors and associated cofactors (Yan, Naltner et al. 2001), nuclear factor 1, members of the AP-1family, and BR22. It has been suggested that NKX2-1plays a central role in the stabilization of these complexes.
*In vitro* NKX2-1 activates transcription of cotransfected *Tg* and *TPO* promoters in non thyroid cells (Damante and Di Lauro 1994), which suggests that NKX2-1 is important for the transcriptional activation of thyroid specific genes. Interestingly, the presence of the NKX2-1 protein does not invariably correlate with active transcription of the *Tg* and *TPO* genes in thyroid development (Damante G, Tell G et al. 2001). From embryonic day 8.5 (E8.5) on, NKX2-1 is present in the thyroid bud together with the other thyroid transcription factors TTF-2 and Pax-8, whereas Tg and TPO do not appear before E13. In lung cells it is responsible for the production of the surfactant proteins and in the hypothalamus NKX2-1 acts as transcriptional regulator of genes encoding neuromodulators and hypophysiotrophic peptides, controlling circadian oscillations in GnRH gene transcription (Matagne, Kim et al. 2012).

Gene-inactivation experiments have revealed some important functions of *Nkx2-1 in vivo*. Nkx2-1 null mice die at birth. They are characterized by impaired lung morphogenesis, lack of thyroid and pituitary, and severe alterations in the ventral region of the forebrain (Kimura, Hara et al. 1996). Other studies, performed at earlier stage of development demonstated that in *Nkx2-1*$^{-/-}$embryos the thyroid *primordium* forms in its correct position but subsequently undergoes degeneration and eventually disappears. At E10.5 the thyroid primordium already appears much smaller in size in comparison with wild type, and at E11 no thyroid cells are detectable; these data and the presence of apoptotic cells suggest that Nkx2-1 is required to prevent the initiation of an apoptotic process (Kimura S, Ward JD et al. 1999). Hence, *Nkx2-1* is essential for the survival of thyroid cells precursor, but it is not necessary for their initial formation. The *Nkx2-1*$^{-/-}$ mouse has been of great relevance because it demonstrates two important concepts: the first is that athyreosis might be due to the inability of the TFC precursors to survive rather than to lack of their specification*;* the second consists in the proof of the concept that athyreosis can be due to a single, heritable genetic lesion. Nkx2-1 is also implicated in epithelial/mesenchymal signaling. In *Nkx2-1*$^{-/-}$ mice a reduction of the number of cartilage rings of the trachea (Minoo P, Su G et al. 1999) was observed. Because there is no Nkx2-1 in the cartilage, it is very likely that the tracheal rings defect is the result of a defective signaling process, which is normally controlled by Nkx2-1 in the tracheal epithelium and which is necessary for the normal development of tracheal cartilage. These interactions represent a necessary step in the morphogenesis of trachea and lungs (Yuan B, Li C et al. 2000). Supporting this hypothesis are studies carried out on defective anatomical structures in *Nkx2-1*$^{-/-}$ mice, showing that the expression of some well-known signaling molecules is controlled by this transcription factor. Bone morphogenetic protein (Bmp)4, a TGFβ-related peptide growth factor, expressed in the growing tip of the branching lung epithelium in a normal embryo, is undetectable in the lungs of *Nkx2-1*$^{-/-}$ embryos; hence, Nkx2-1 controls, directly or indirectly, *Bmp4* expression, and it is very likely that the absence of this growth factor is responsible for the alteration in lung morphogenesis in the mutant.

Because the initiation of *Nkx2-1* expression is coincident with specification, elucidation of these mechanisms might shed some light on the specification process itself. In mice, an inductive signaling by the axial mesendoderm (Shimamura K and Rubenstein JL 1997) could be implicated in the initial activation of *Nkx2-1* in the prosencephalic neural plate. Sonichedgehog (Shh) is relevant for the ventralizing signal, and *Nkx2-1* is expressed in patterns that are either coincident or adjacent to domains of *Shh* expression. Analysis of *Shh*$^{-/-}$ embryos has revealed that *Nkx2-1* is indeed regulated by this factor only in the forebrain. Indeed, in *Shh*-deficient embryos no Nkx2-1 is observed in the brain, whereas normal levels of the protein are detected in the thyroid and lung anlage (Pabst O, Herbrand H et al. 2000). On the basis of *in vitro* studies, it was proposed that the zinc finger factor Gata6 regulates the transcription of

*Nkx2-1* However, analysis of *Gata6*$^{-/-}$ chimeric lungs has demonstrated that Nkx2-1 is localized normally in epithelial cells of both wild type and mutated lungs *(Keijzer R, Van Tuyl M et al. 2001)*. In conclusion, Nkx2-1 appears as a protein that is highly regulated in a strict cell-specific manner, because it regulates different genes in different cell types. Furthermore, some genes appear to depend on Nkx2-1 for expression in some cells, whereas they are independent from it in others, as in the case of *Bmp4* in lung and posterior pituitary. In the thyroid cells themselves, Nkx2-1 clearly plays radically different roles during development, as it controls survival at the beginning of organogenesis and the expression of TFC-specific genes in adult life.

Early studies searching for *NKX2-1* mutations in humans affected by CH were disappointing. In viable newborns, it could be difficult to find homozygous loss-of function mutations in the *NKX2-1* gene; the essential role of Nkx2-1 in lung and brain development, assessed in the animal model, made it possible to anticipate that such mutations should cause death immediately after birth. Subsequently, a heterozygous deletion encompassing the *NKX2-1* locus in an isolated infant (Devriendt, Vanhole et al. 1998) and in two siblings (Iwatani, Mabe et al. 2000) was reported. Finally, the analysis of some large families (Breedveld, van Dongen et al. 2002) provides strong evidence that *NKX2-1* defects are directly responsible for benign hereditary chorea, an autosomal-dominant movement disorder. When tested *in vitro*, the corresponding mutated forms of Nkx2-1 show neither functional activity nor a dominant-negative effect on the wild-type form. These data suggest that the haploinsufficiency is responsible for the pathological phenotype. On the contrary, *Nkx2-1*$^{+/-}$ mice are considered normal, on the basis of anatomical and morphological studies (Kimura, Hara et al. 1996). *NKX2-1* mutations have been described in several patients with primary CH, respiratory distress and benign hereditary chorea, which are manifestations of the "Brain-Thyroid-Lung Syndrome" (BTLS, OMIM # 610978) (Table 7). In the majority of cases haploinsufficiency has been considered to be responsible for the phenotype (Ferrara, De Michele et al. 2008). Only few mutations produce a dominant negative effect on the wild type *NKX2-1* (Guillot, Carre et al. 2010), (23Nettore, Ferrara et al. 2013) and among those in two cases it has been reported a promoter-specific dominant negative effect (Moya, Perez de Nanclares et al. 2006), (Nettore, Ferrara et al. 2013). All the published *NKX2-1* mutations have variable functional effects, even if the mutations occur in similar regions of the protein. The clinical features of the patients carrying *NKX2-1* mutations are reported in Table 7. Phenotypes are very variable and there is no correlation between the clinical manifestations and the molecular alterations (Ferrara, De Michele et al. 2008). Both the incomplete penetrance and the variability of the phenotype could be due to the effect of other modifier genes as well as of environmental factors. Furthermore, when the disease is associated with a large chromosomal deletion, we cannot exclude that the loss of other genes is contributing to variability of the phenotype.

### 1.4.2 *PAX8*

PAX8 is a transcription factor belonging to the mammalian family of PAX proteins, characterized by the presence of a 128 aminoacid DNA binding domain (paired domain, Prd). Within this family, composed of nine members (Mansouri, Chowdhury et al. 1998) *Pax8*, on the basis of a higher sequence similarity, forms a subfamily with *Pax2* and *Pax5 (Walther C, Guenet JL et al. 1991)* (Dorfler P and Busslinger M 1996). The human gene maps to chromosome 2q12-q14 encoding for *PAX8* protein of 450 aminoacids even if different isoforms have been characterized for alternative splicing. The Full-length form, Pax8a, consists of 12 exons and is the most protein species present. The Pax8b isoform lacks exon 9, while the isoform Pax8c presents a frameshift of reading from exon 9, which generates a region rich in proline and termination premature transcript (Poleev A, Wendler F et al. 1995). Several mechanisms may be involved in regulation of the levels of cytoplasmic protein such as sumoylation and the inactivation by the redox state (De Felice M, Ovitt C et al. 1998), (Cao, Kambe et al. 2005).
*Pax8* is expressed in alla stages of thyroid development and in adulthood. In the nervous system, *Pax8* mRNA is transiently expressed in the myelencephalon and through the entire length of the neural tube. No signals are detected in the brain at later stages of the development, nor are they present in the adult brain. In the excretory system, *Pax8* mRNA is present in the nephrogenic mesenchyme, which gives rise to the epithelial structures of nephrons as a consequence of the instructive interactions of the growing nephric duct and ureter.
Analysis of *Pax8*$^{-/-}$ mice revealed the role of this transcription factor during embryonic life. Whereas no phenotype has been detected in heterozygous *Pax8*$^{-/-}$ mice, homozygous *Pax8*$^{-/-}$ mice are alive at birth but show growth retardation and die within 2-3 weeks if not treated with thyroid hormones (Mansouri, Chowdhury et al. 1998). These mice do not display any apparent defects in the spinal cord, midbrain/hindbrain boundary, or kidneys. On the contrary, the thyroid gland in these mice is severely altered and no follicles and TFC can be detected, while calcitonin-producing C cells are present. Hypothyroidism is the cause of death of the mutated animals: the administration of T4 to *Pax8*$^{-/-}$ mice allows the animals to survive. A detailed study during the early steps of thyroid morphogenesis shows that in *Pax8* null embryos the thyroid diverticulum is able to evaginate from the endoderm, but Pax8 is required for further development. In the absence of this transcription factor, at E11.5, thyroid *primordium* appears smaller (hypoplastic thyroid) than in wild-type mice, and at E12.5 follicular cells are essentially undetectable, indicating that Pax8, similarly to Nkx2-1, is required for the survival of thyroid cell precursors but not for their specification (Pasca di Magliano M, Di Lauro R et al. 2000). Furthermore, in the thyroid anlage of *Pax8*$^{-/-}$ mice the expression of *Foxe1* and *Hhex* is strongly down-regulated (Parlato R, Rosica A et al. 2000). In addition to these important roles in morphogenesis of the TFC

component of the thyroid gland, it has been shown, in cell culture systems, that *Pax8* is a master gene for the regulation of the thyroid-differentiated phenotype.
In conclusion, Pax8 not only is required for the survival of the thyroid precursor cells but also holds a specific upper role in the genetic regulatory cascade, which controls thyroid development and functional differentiation. These functions of Pax8 in thyroid development are consistent with the findings that in other organs *Pax* genes have a relevant role both in initiating and maintaining the tissue-specific gene expression program (Nutt SL, Heavey B et al. 1999), (Bouchard M, Souabni A et al. 2002).

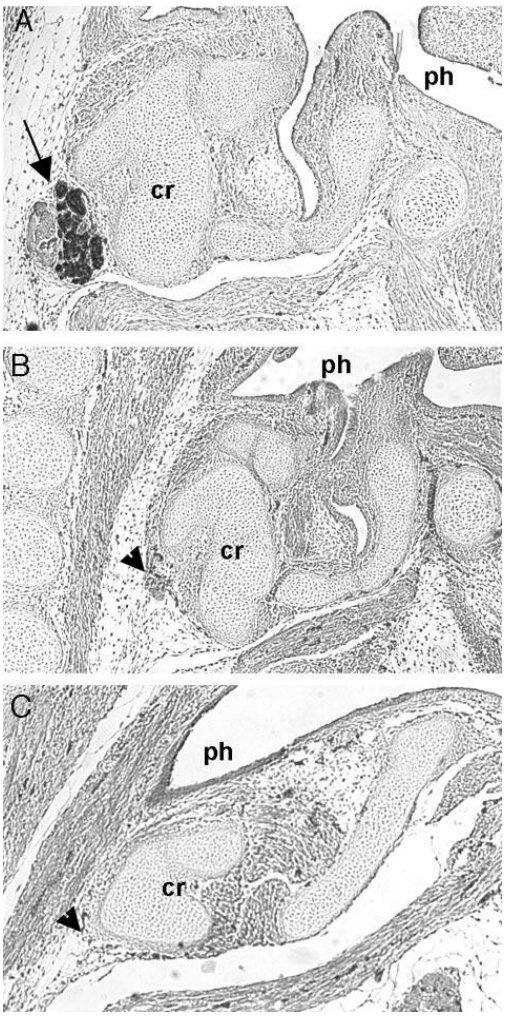


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**Fig. 3**. Both Titf1 and Pax8 are required for thyroid development. Sagittal sections of wild-type (A), *Pax8*$^{-/-}$ (B), and *Titf1/Nkx2-1*$^{-/-}$ C) E15.5 mouse embryos stained with an anti-Titf1/Nk2-1 antibody are shown. In wild-type embryo, the developing thyroid (*arrow*) is positioned dorsal to the cricoid cartilage. In both the mutated embryos the thyroid tissue is undetectable (*arrowhead*).

The involvement of *PAX8* has been described in sporadic and familial cases of CH with TD (Table 6). So far, loss-of-function mutations in the DNA binding domain of *PAX8* have been identified in two subjects with isolated CH and in the affected members of four families (Table 6); when tested in a transfection assay, these mutated proteins are unable to drive transcription from *TPO*

promoter, a target of this transcription factor. All affected individuals are heterozygous for the mutations and, in the familial cases, transmission is autosomal dominant. The different phenotypes described varied from mild to severe hypoplasia of the thyroid also among the affected members of the same family (Macchia, Lapi et al. 1998). Furthermore, the same heterozygous mutation been detected in both a patient with CH hypoplasia and in her mother with no signs of hypothyroidism (Congdon T, Nguyen LQ et al. 2001), indicating incomplete penetrance. Even if, in the majority of cases CH is due to thyroid hypoplasia, three patients with athyreosis and two with thyroid ectopy have been described (Table 6). However, to explain the severe hypoplasia described in some patients, the mutant gene should be preferentially expressed. Moreover, such monoallelic expression of *PAX8* should be a specific mechanism in humans and could be a explanation of this phenomenon. Alternatively, the TD observed in $PAX8^{+/-}$ patients could be due to a gene dosage requirement (haploinsufficiency). This indicates that in humans, contrary to mice models, both *PAX8* alleles are necessary for correct thyroid morphogenesis, and a reduced dosage of the gene product (haploinsufficiency) causes dysgenesis. This phenomenon has already been observed in other systems (Bi W, Huang W et al. 2001), (Lindsay EA, Vitelli F et al. 2001) included for other *Pax* genes (van Raamsdonk CD and Tilghman SM 2000), (Wilm B, Dahl E et al. 1998). The protein acts properly within a concentration range that is sensitive to a 2-fold change. The knockout of the gene, in mice, leads to the disappearance of the thyroid primordium, whereas the reduced amount of Pax8 in these patients is not sufficient to support the normal development of the gland. The influence of other modifier genes could explain the variable penetrance of the phenotype. According to this hypothesis, the discrepancy between humans and mice could be related to the genetic background of the mouse line used in generating the corresponding animal models.

The molecular mechanisms by which PAX8 mutations lead to TD are still unclear. A recent work describes and functionally characterizes two novel PAX8 mutations leading to TD by two different mechanisms. Both mutations (S54R and R133Q) determined a significant reduction in the transcription induction of thyroglobulin and thyroperoxidase promoters, but while the S54R mutant presented an impaired DNA binding, the DNA-binding properties of R133Q mutation are not affected and the R133Q has no dominant negative effect on the WT protein *in vitro (Hermanns, Grasberger et al. 2013).*

Hemiagenesis of the thyroid is a frequent observation in mice double heterozygous for $Nkx2\text{-}1^{+/-}$ and $Pax8^{+/-}$ *(Amendola, De Luca et al. 2005)* but, to date, in humans no genetic alterations have been found in patients with thyroid hemiagenesis.

### 1.4.3 *FOXE1*

FoxE1, also called TTF-2, is a transcription factor containing a forkhead domain and a polialanine stretch of variable length (Macchia, Mattei et al. 1999; Macchia 2007; Santarpia, Valenzise et al. 2007). While *Foxe1* is located on mouse chromosome 4, the human gene maps to chromosome 9q22 and encodes for a 42 kDa protein.

*Foxe1* has a wider domain of expression. In ectoderm-derived structures, at an early stage of development, Foxe1 is present in the posterior stomatodeum, in the buccopharyngeal membrane, and in the cells of the roof of the oral cavity indenting to constitute Rathke's pouch, which will form the various components of the anterior pituitary. At later stages, *Foxe1* mRNA expression in the pituitary is down-regulated (Zannini, Avantaggiato et al. 1997), whereas it appears in the secondary palate, in the definitive choanae, and in the whiskers and hair follicles (Dathan N, Parlato R et al. 2002). In the adult, Foxe1 is still present in the thyroid, whereas the expression in the esophagus is faint.

In humans, *FOXE1* mRNA is also detected in adult testis (Clifton-Bligh RJ, Wentworth JM et al. 1998) and several other tissues (Chadwick BP, Obermayr F et al. 1997). However, in the latter case, additional mRNAs of different size have been reported. Homozygous *FoxE1* knock-out mice are born at the expected ratio but die within 48 h. These mice have no thyroid in its normal location and absence of thyroid hormones. Furthermore, the mice show a severe cleft palate, probably responsible for the perinatal death, and elevated TSH levels in the bloodstream (De Felice M, Ovitt C et al. 1998). Studies of the early stages of thyroid morphogenesis demonstrate that the budding of the thyroid primordium does not require Foxe1, because the anlage can be easily detected by the expression of *Nkx2-1* and *Pax8*, and a normal primordium is formed. However, at E9.5 in *Foxe1* null embryos, thyroid precursor cells are still on the floor of the pharynx, whereas in wild-type embryos they are detached from the pharynx cavity and begin to descend (Fig 4). At later stages of development, in the absence of *Foxe1*, mutant mice exhibit either a small thyroid remnant still attached to the pharyngeal floor or no thyroid gland at all. The variable expressivity of the phenotype could be due to stochastic events during thyroid morphogenesis. In addition, it is possible that either the individual genetic background or sex-related factors are responsible for the variability of the phenotype of the Foxe1 null mice. It is worth noting that the non-migrating thyroid cells are able to complete their differentiation process as demonstrated by the presence of thyroglobulin. Hence, *FoxE1* plays an essential role in the control of the migration of TFC precursors whereas both Nkx2-1 and Pax8 seem to be relevant in the survival and/or differentiation of these cells. Furthermore, the data that found that in 50% of *Foxe1* null mice the thyroid disappears indicate that this gene, too, is implicated in the control of the survival of thyroid cells at a step different from those controlled by Nkx2-1 and Pax8. The role of Foxe1 in the adult gland is still a matter of study.

Functional studies in cell cultures have demonstrated that Foxe1 can act as a promoter-specific transcriptional repressor (Perrone L, Pasca di Magliano M et al. 2000). Because the *Foxe1* null mice die at birth, only the creation of an animal model with a thyroid-specific, conditional knockout of *Foxe1* will permit elucidation of the role of this factor in the physiology of the gland.

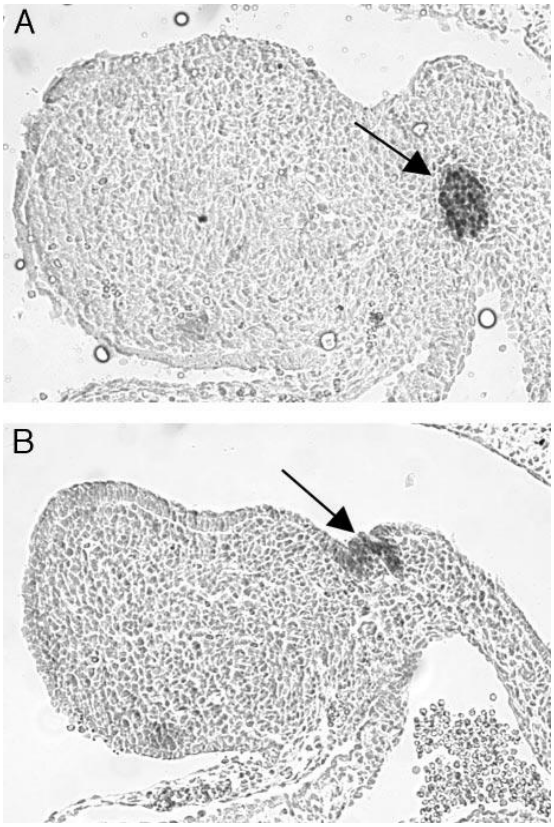


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**Fig. 4.** Foxe1 is required for thyroid precursor cells migration. Sagittal sections of *Foxe1*-/- (A) and *Foxe1*-/- (B) E11 mouse embryosstained with an anti-Titf1/Nk2-1 antibody. The *arrows* point to the thyroid bud. In the mutated embryo, the thyroid bud is still on the floor of the primitive pharynx.

*FOXE1* within its coding sequence contains a polyalanine tract of variable length, ranging from 11 to 19 alanines (Amiel, Trochet et al. 2004; Carre, Castanet et al. 2007). The studies of other genes containing polyalanine stretches demonstrated that changes in polyAla result in a less severe phenotype when compared to causative point mutations at the same locus

(Amiel, Trochet et al. 2004). A few studies have pointed to the potential role of *FOXE1*-polyAla length polymorphism in determining the susceptibility to TD (Carre, Castanet et al. 2007), (Hishinuma, Ohyama et al. 2001), (Tonacchera, Perri et al. 2004).
A recent study, report that the length of *FOXE1*-polyAla varied from 12 to 18 alanines, compared to previuos reports. In the population studied, in the patients with the familial form of TH, a significantly higher incidence of longer variants (>16 codons) of *FOXE1*-polyAla was noted in comparison to the control group as well as the patients with the sporadic form of TH. All patients with the familial form were heterozygous or homozygous for the longer variant of *FOXE1*-polyAla. The length of *FOXE1*-polyAla in the patients with the sporadic form of TH and the control subject did not differ significantly (Szczepanek E 2011).
Homozygous mutations in *FOXE1* gene have been reported in patients affected by Bamfort's syndrome. This syndrome is characterized by cleft palate, bilateral choanal atresia, spiky hair and athireosis (Bamforth JS, Hughes IA et al. 1989). Even if defects in choanae and hair follicles have not yet been investigated in *Foxe1* null mice, *Foxe1* is expressed in both these structures (Dathan N, Parlato R et al. 2002). Indeed, two homozygous mutations in *FOXE1* gene have been described in two pairs of siblings affected by this syndrome (Clifton-Bligh RJ, Wentworth JM et al. 1998), (Castanet M, Park SM et al. 2002). All the affected members carry homozygous missense mutations in conserved amino acids of FOXE1, and the mutant proteins, when tested *in vitro*, show a reduction in both DNA binding and transcriptional activity. While in mice the absence of *FoxE1* causes either athyreosis or ectopia (De Felice M, Ovitt C et al. 1998), in humans, *FOXE1* mutations have never been associated to the thyroid ectopy.

### 1.4.4 *NKX2-5*

In addition to *Nkx2-1*, other genes of the Nkx2 family are present in the primitive pharynx and the thyroid anlage, such as *Nkx2-3*, *Nkx2-5*, *Nkx2-6*. Nkx2-5, which is a homeodomain-containing protein, originally identified as a potential vertebrate homologue of the *Drosophila* gene *tinman (Komuro and Izumo 1993).* The gene encoding NKX2-5 in humans is located on chromosome 5q34 and consists of two exons encoding for a 324 aminoacids protein. Nkx2-5 plays a crucial role in heart morphogenesis (Chen and Schwartz 1995). Its transcripts are found in the heart (Kasahara, Lee et al. 2000), in heart progenitor cells (Lints TJ, Parsons LM et al. 1993), in mammary gland during lactation (Dentice, Luongo et al. 2004), and in the thyroid primordium during development (Kasahara, Bartunkova et al. 1998). In *Nkx2-5*$^{-/-}$ embryos the thyroid bud is smaller when compared to the wild type, suggesting that Nkx2-5 is required for thyroid development (Dentice, Cordeddu et al. 2006). In humans, NKX2-5 is essential for normal heart

morphogenesis, myogenesis, and function (Tanaka M, Schinke M et al. 2001), and several loss of function mutations in NKX2-5 have been described in patients with congenital heart diseases (Hirayama-Yamada, Kamisago et al. 2005). Transmission is autosomal dominant, with incomplete penetrance (Benson, Silberbach et al. 1999; Tanaka T, Inazu T et al. 1999).
Heterozygous mutations in *NKX2-5* have been associated to the human ectopic thyroid (Dentice, Cordeddu et al. 2006). Functional studies demonstrated that these mutants exhibited a significant functional impairment, with reduction of transactivation properties and dominant negative effect (Table 8). Because Nkx2-5 acts as a homodimer through its homeodomain, dimerization is impaired by some disease-causing mutants that are dominant negative over the wild-type counterpart. Recently, it has been reported a girl with athyreosis presenting heterozygous mutations in both *NKX2-5*, and *PAX8* genes (Hermanns, Grasberger et al. 2011). Haploinsufficiency, monoallelic expression, or imprinting could all have an impact on the phenotype (Vassart and Dumont 2005) and again patients carrying *NKX2-5* mutations show a phenotypic variability in both heart and thyroid phenotype. However, the role of Nkx2-5 in thyroid development remains poorly understood.

### 1.4.5 *TSHR*

In late stage of thyroid organogenesis, at E15.5, the thyrotropin receptor (Tshr) starts to be expressed in TFC. Tshr is a protein of 765 aminoacids long both in humans and in mice and belongs to the superfamily of G protein-coupled receptors. TSH binds to the extracellular portion of the receptor, a long amino-terminal extracellular domain that includes a succession of leucine-rich repeats. The COOH portion of Tshr forms the transmembrane and intracellular domains involved in transducing signals (Parmentier M, Libert F et al. 1989), (Misrahi M, Loosfelt H et al. 1990). Tshr, localized on chromosome 14q31 in humans (Rousseau-Merck MF, Misrahi M et al. 1990), (Libert F, Passage E et al. 1990) and chromosome 12 in mice (Taylor BA, Grieco D et al. 1996), spreads over 60 kb and is split into 10 exons. The extracellular amino-terminal domain is encoded by nine exons, whereas the transmembrane domain and cytoplasmic tail are encoded by a single large exon (Gross B, Misrahi M et al. 1991). It is interesting to note that other G protein-coupled receptors (such as adrenergic or muscarin receptor genes) are devoid of intron; therefore, Tshr seems to have evolved from an intronless protoreceptor fused to a set of duplicated genes coding for a leucine-rich sequence. The expression of Tshr mRNA is detected in rat thyroid at E15 (Brown RS, Shalhoub V et al. 2000) (corresponding to E13.5–E14 in mice) and strongly increases by E17. Hence, Tshr mRNA is detected in the developing thyroid after the completion of the migration of the primordium, before the first evidence of follicular organization in the gland. The way the activation of Tshr regulates both proliferation and functioning of adult thyroid cells has already been exhaustively reviewed (Dumont JE, Lamy

F et al. 1992), (Kimura T, Van Keymeulen A et al. 2001). The analysis of thyroid development in mice carrying spontaneous (Beamer, Eicher et al. 1981) or induced (Marians, Ng et al. 2002) alterations in the Tshr gene has provided a powerful tool in the exploration of the role of the TSH/Tshr pathway during embryonic life. The role of the TSHR in thyroid differentiation was first identified in *Tshr*$^{hyt/hyt}$ mice, affected by primary hypothyroidism with elevated TSH and hypoplastic thyroid, as a consequence of a loss of function mutation in the fourth transmembrane domain of TSHR (pro556Leu), which abolishes the cAMP response to TSH (Stuart, Oates et al. 1994). As the Tshr$^{hyt/hyt}$ mice, also the Tshr null mice display a severe hypothyroidism, associated with thyroid hypoplasia in adult life. However, at birth, in both these mutants, the size of the thyroid does not appear to be affected, and the gland displays only some alterations in its structure. A detailed analysis performed at the end of the organogenesis, at E17, has revealed that in the absence of a functional Tshr, the size and the follicular structure of the thyroid are not affected, and the amount of Tg does not change, whereas the expression of both TPO mRNA and NIS is strongly down-regulated (Postiglione MP, Parlato R et al. 2002). These data indicate that, during embryonic life, the TSH/Tshr signaling is required to complete the differentiative program of the TFC, but, unlike what happens during adult life, this signaling is not relevant in controlling the growth of the gland.

The first genetic errors associated with CH with TD have been identified in the gene coding for TSHR. In 1968, Stanbury et al. observed that TSH unresponsiveness could be a cause of CH in the absence of goiter. The identification of Tshr$^{hyt/hyt}$ mice (Beamer, Eicher et al. 1981), affected by a primary hypothyroidism with elevated TSH and hypoplastic thyroid, offered a useful model for this autosomal-recessive form of CH. The first mutations were identified in three siblings (Sunthornthepvarakui, Gottschalk et al. 1995)(194) characterized by high TSH and normal thyroid hormone levels in the serum. The siblings were compound heterozygous, carrying a different mutation in each of the two alleles, one allele derived from each parent. After this report other mutations in the *TSHR* gene have been identified in patients affected by CH with thyroid hypoplasia and increased TSH secretion. The different phenotypes described range from asymptomatic hyperthyrotropinemia to severe CH with a profound hypoplasia of the thyroid. Part of the variability of the phenotype can certainly be explained by the diverse residual activity of the mutated TSHR molecules. However, the affected members of the same family show occasionally diverse expressivity of the hypothyroid phenotype, too, thus suggesting that other genes are capable of influencing the TSHR activity. Until now, patients with ectopic thyroid have never been described. This is expected because the TSHR-induced pathway is not involved in the migration of the embryonic thyroid. Subjects heterozygous for loss-of-function mutations in the *TSHR* genes are euthyroid; in the familial forms, consistently, the diseasis inherited as an autosomal-recessive trait. However, in many heterozygous

relatives of the affected members, the serum TSH values fluctuate above the upper limit of the normal range (Russo D, Betterle C et al. 2000).

### 1.4.6 Other genes-*DNAJC17*

TD is a multigenic disorder. This hypothesis is consistent with the finding that there is no clear Mendelian transmission of TD in families with more than one affected member (Castanet, Polak et al. 2001). Furthermore, in familial cases of TD associated with mutations in either the *NKX2-1* or *PAX8* gene, the disease is characterized by incomplete penetrance and variable expressivity, thus suggesting the contribution of additional genes to the phenotype (De Felice M and Di Lauro R 2004). The study of multigenic diseases in humans is difficult. Studies on mouse models indicate that the pool of genes, mostly transcription or growth factors, involved in thyroid development is rather large, and defects in these genes or in their yet unidentified targets could be relevant for the pathogenesis of TD in humans. It is known that, neither *Pax8* nor *Nkx2-1* heterozygous null mice show overt thyroid defects. But, the combination of two heterozygous null mutations, in *Nkx2-1* and *Pax8*, results in a severe hypothyroidism characterized by thyroid hypoplasia and increased incidence of thyroid hemiagenesis. This model has been named DHTP, for double heterozygous for *Nkx2-1* and *Pax8*. Interestingly, the severe hypothyroidism is present only in the C57BL6/J (B6) genetic background, whereas it is completely absent in both 129/SvPasCrl (Sv) and DHTP/B6-Sv F1 hybrids. These data suggest that homozygosity for B6-specific alleles, in the presence of null mutations in both *Nkx2-1* and *Pax8*, is involved in the emergence of TD. Genetic linkage analysis in a DHTP backcross population identified two potential loci linked to the hypothyroid phenotype, with one of them having the major effect. On the basis of a single-nucleotide polymorphism (SNP) that causes a non synonymous aminoacid change in a highly conserved protein domain between B6 and Sv strains, it was identified *Dnajc17*, as a candidate modifier gene for hypothyroidism. Indeed, Dnajc17 presents a phenylalanine residue at position 273 in the B6 mice, but it shows a tyrosine at the same position in all other mouse strains. Even though the phenylalanine-tyrosine change is a conservative missense mutation, tyrosine273 is conserved in *Dnajc17* ortholog genes throughout the evolution from metazoans (Fig. 5). It is worth noting that in humans, disease-associated mutation soccur often at sites that are conserved in evolution. In addition, analysis performed with NetPhos 2.0 software predicts that tyrosine$^{273}$ is very likely to be phosphorylated, suggesting that the absence of this post translational modification could be responsible for an impaired function of the protein.
The gene coding for DNAJC17 maps on chromosome 2 of mouse and human chromosome 15. It consists of 11 exons and encodes a protein of 304 amino acids, belonging to the family of heat-shock-protein-40 type III. *Dnajc17* is expressed from the early stage of embryonic life in the developing thyroid

(E10.5) (Fig.6) and remains expressed in the adult thyroid as well as in differentiated thyroid follicular cells in culture. In addition, the findings that *Dnajc17* is expressed in thyroid and it is localized in the nucleus are consistent with a role of this protein in modulating the transcription of some thyroid-specific genes. Studies in vivo of this protein demonstrated that Dnajc17$^{+/-}$ mice appeared normal, while Dnajc17$^{-/-}$ pups were never observed postnatally, indicating that theDnajc17 null mutation is recessive embryonic lethal. Furthermore it found Dnajc17$^{-/-}$ two-cell embryos, but no homozygous embryos were recovered after the morula stage. These findings indicate that Dnajc17$^{-/-}$ embryos were developmentally arrested before implantation and indicate an essential role of this gene in early mouse development. An additional aspect that support the candidacy of Dnajc17 as a modifier gene of the DHTP phenotype is the observation that the hypothyroid phenotype in this model is associated with an impaired transcription of the Tg gene, well known target of Nkx2-1 and Pax8. Consistent with this are the data indicating that Dnaj proteins interact, via a highly conserved domain (J domain), with Hsp70 chaperone proteins and regulate the activity of these chaperones (Qiu, Shao et al. 2006). It has been reported that chaperone proteins can control the disassembly of transcriptional complexes (Freeman and Yamamoto 2002) and can act as regulators in gene expression processes in Drosophila melanogaster as well as in mammalian cells. Hsp70proteins, the partners of Dnaj, are involved in a number of biological processes, and since Dnajc17 has a nuclear localization, disturbances in Hsp40-Hsp70 interactions could have pleiotropic effects, including alterations in the regulation of the transcriptional activity of Nkx2-1 e/o Pax8.

| **Species** | **Alignment** |
|---|---|
| *Mus muris* (B6 strain) | RD**F**ESLVMMRMRQAAERQQLIAQMQQED—EGRPT |
| *Mus muris* (Sv strain) | RD**Y**ESLVMMRMRQAAERQQLIAQMQQED—EGRPT |
| *Rattus norvegicus* | RD**Y**ESLVMMRMRQAAERQQLIAQMQQED—EGRPT |
| *Homo sapiens* | RD**Y**ESLVMMRMRQAAERQQLIARMQQEDQ–EGPP |
| *Macaca mulatta* | RD**Y**ESLVMMRMRQAAERQQLIARMQQEDQ–EGPPT |
| *Monodelphis domestica* | RD**Y**ESLVMMRMRQAAERQQLIEQMQREDE–EGLPT |
| *Danio rerio* | RD**Y**ESVVLMRLRQAEERKRLIEQMKREEEDDTA |
| *Anophelesgambiae* | RD**Y**ESLVLRQLRQAEERKRLIEQMMKEEAEAEGETTAQ |
| *Drosophila melanogaster* | TD**Y**EDLVMRKLRQAEERKRLIEQMMKDE—EGE |

**Fig. 5.** Alignment of the deduced amino acid sequence of the C-terminal region of Dnajc17 orthologous genes among different species. Tyrosine 273 residue is in *bold.*

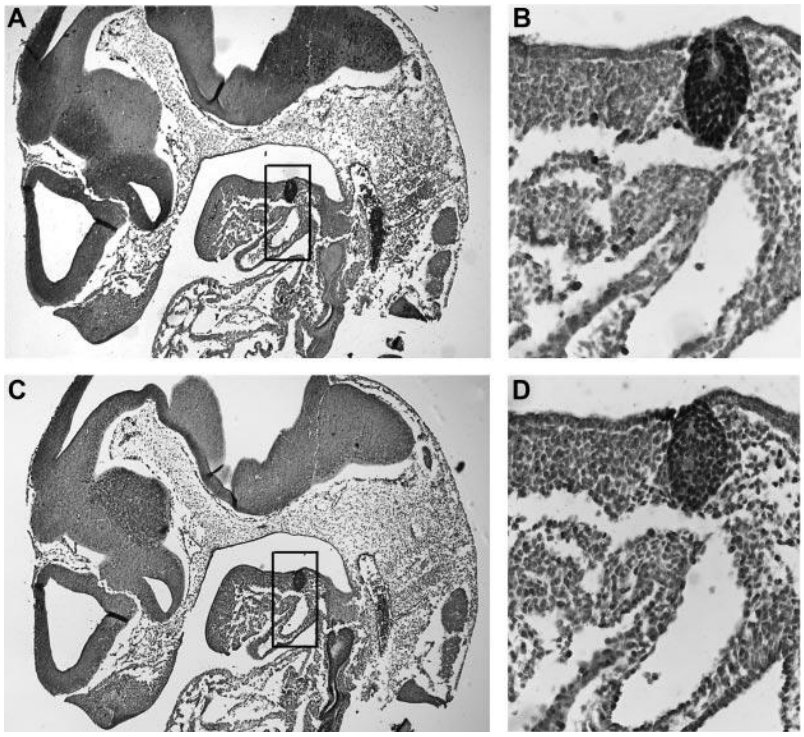


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**Fig. 6.** Dnajc17 expression in mouse embryo. A and C, Sagittal sections of E10.5 wild type mouse embryo were immunostained with an anti-Dnajc17 (A) or anti-Nkx2-1 (C) specific antibody (magnification x50). B and D, Higher magnification (x200) of the *boxed areas* in A and C, respectively. The expression of Nkx2-1 is used as a molecular marker to identify the thyroid bud. Dnajc17 has a robust expression in developing thyroid

# 2. AIM OF THE STUDY

The objective of the this work was to study the role of thyroid transcription factor (*NKX2-1, NKX2-5, PAX8*) already known to be involved in thyroid organogenesis as well as the possible association of thyroid dysgenesis (TD) with other genes not previously linked to the disease in humans.

In the last years, several genes playing a major role in thyroid development have been identified. Alterations of the thyroid morphogenesis have been associated with disturbances in the function of some of these genes, suggesting that genetic mechanisms can be responsible for TD.

Several mutations in *NKX2-1, NKX2-5, FOXE1* and *PAX8* genes, have been found in patients with congenital hypothyroidism (CH) due to TD. In particular, *NKX2-1* mutations were associated with the "Brain-Thyroid-Lung Syndrome", which combines Congenital Hypothyroidism (CH), neurological symptoms (hypotonia evolving to benign hereditary chorea (BHC) after infancy), and pulmonary symptoms.

The phenotypic variability and incomplete penetrance observed in familial cases of patients with TD carrying mutations in either *NKX2-1* or *PAX8* genes supported the possibility that other interacting genes may modulate the phenotype. Among these, it has been demonstrated that *Dnajc17* is highly expressed in the thyroid bud and has an essential function during development.

In order to better understand the complexities of the genotype–phenotype correlation in TD and to identify novel genetic alterations in patients with TD, we sequenced the entire coding region of *NKX2-1* in a family from Curitiba, affected by Brain-Lung-Thyroid Syndrome. We also screened by SSCP and HRM analysis known genes (*NKX2-1, NKX2-5, PAX8),* and *DNAJC17*, a new candidate gene, in 76 patients with persistent CH.

# 3. MATERIAL AND METHODS

## *3.1 Patients*

Our study included 76 patients with persistent CH identified by the Italian screening program for congenital hypothyroidism.
The group was composed as follows: 17 patients had agenesis; 19 hemiagenesis; 31 ectopy; 4 hypoplasia of the thyroid gland and 5 patients CH with normal thyroid. 100 normal volunteers with no thyroid abnormalities were used as controls.
In addition we had the opportunity to study a family living in Curitiba, Brazil with five affected patients showing the clinical manifestations of brain–thyroid–lung syndrome (BTLS) (Fig. 7). Clinical informations are reported in the next paragraph.
Informed consent was obtained from all the studied patients or their parents.

## *3.2 The "Brazilian Family"*

The family included five affected patients in three generations (Fig. 7).

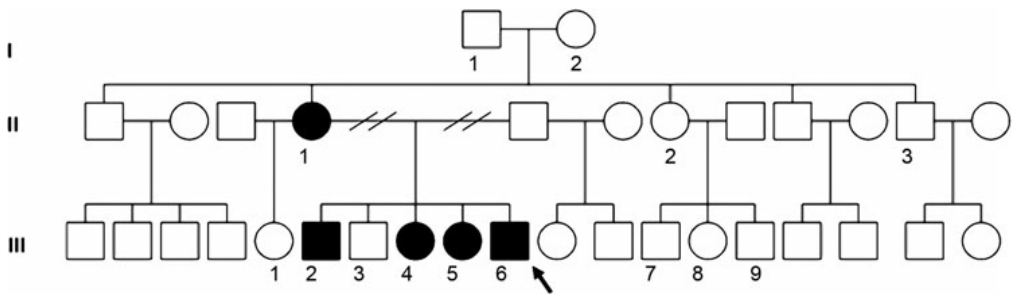


**Fig.7** Pedigree of the studied family. The family members carrying the c.493delC mutation (p.Arg165Glyfs*32) and showing the clinical manifestation of brain–thyroid–lung syndrome (BTLS) are in black. The proband (III-6) is indicated by an arrow.

Collection of clinical information was difficult because the members of the family live in different towns; therefore, some of the clinical characteristics were missing. The proband (III-6) was the fifth child of non consanguineous parents. He was born at term with a birth weight of 2820 g, but a delay in motor development was soon evident: he took his first steps at 24 months of age. During infancy, the proband showed moderate generalized choreiform movements and cerebellar ataxia that remained stable thereafter, accompanied by several episodes of respiratory insufficiency that were diagnosed either as asthma or pneumonia. Routine blood chemistry and hematology tests and electrocardiograms were normal. Brain MRI, electromyography, and muscle biopsy were also normal. When the proband was 13 years old, his thyroid function tests (TFTs) revealed subclinical hypothyroidism: serum thyrotropin (TSH) was 9.1mU/L (normal range 0.35–4.94 mU/L), free thyroxine (FT4) was 1.07 ng/dL (normal range 0.7–1.48 ng/dL) and triiodothyronine (T3) was 127.55 ng/dL (normal range 58–159 ng/dL). Serum antithyroid antibodies tests were negative. The proband's mother (II-1) presented with short stature, mild generalized choreiform movements since infancy, subclinical hypothyroidism (TSH 8.51 mU/L, FT4 1.07 ng/dL, T3109.46 ng/dL), and episodes of respiratory insufficiency (diagnosed as asthma) in childhood. Subject III-2, a brother of the proband, presented with cervical dystonia, moderate generalized choreiform movements, and hypothyroidism (TFT not available) at the age of 22 years. Subject III-4 (the oldest sister of the proband) was diagnosed with a mild choreiform movement disorder at the age of 17 years. At that time, subclinical hypothyroidism (TSH8.13 mU/L, FT4 1.1 ng/dL, T3 122.08 ng/dL) was also noticed. She was treated with 25 lg/d of L-thyroxine, which normalized serum TSH levels. The proband's youngest sister (III-5) presented with moderate generalized choreiform movements, mild cognitive deficit, and subclinical hypothyroidism (TSH 5.43 mU/L, FT41.19 ng/dL, T3 126.60 ng/dL) at the age of 14 years. All the other members in the family (I-1, I-2, II-2, II-3, III-1,III-3, III-7, III-8, III-9) had normal TFTs and normal thyroid morphology on ultrasound examination.

### *3.3 DNA samples*

Genomic DNA was extracted from peripheral blood leukocytes according to the manufacturer's instructions (FlexiGene DNA Kit, Qiagen, Hilden, Germany) and stored at 4°C until used.

*3.4 PCR conditions*

PCR were performed using the thermostable recombinant DNA polymerase (VWR, Geldenaaksebaan, Belgium). PCRs were run in a Thermo Cycler-One Gradient (EuroClone, Milano, Italy). Negative controls were always included. Exons 1 to 3 of the *NKX2-1* gene, exons 3 to 10 of the *PAX8* gene, exons 1 to 5 of the *NKX2-5* gene and exons 1 to 11 of the *DNAJC17* gene were amplified in a 15 -µl reaction using the primer pairs shown in Table 1 to 4. In the tables also the annealing temperature each PCR product are given.
PCR buffer cointaned 10 mM Tris-Hcl pH 8.3, 50 mM KCL, 1,5 mM $MgCl_2$, 10% DMSO, 200 µM dNTPs, The following PCR conditions were used: initial denaturation for 2 min at 95 °C, then 35 cycles with 1 min at 94 °C, 1 min at the temperature specific for each primer pair and 1 min at 72 °C, followed by a 5 min incubation at 72 °C.
The products mixed with a loading dye were run on a 1–1.5% agarose gel (SeaKem LE Agarose, Lonza, Rockland, ME USA) stained with ethidium bromide and visualized under u.v. light with AlphaImager Mini (CellBiosciences, Santa Clara USA).

| Exon | Sequence | Tm | Lenght bp |
|---|---|---|---|
| Ex1F | GTGGCTGCCTAAAACCTG | 60.4°C | 700 bp |
| Ex1R | GGCCGCCCTCCCTGATGC | | |
| Ex2Q | CCAGAACCACCGCTACAAAATGAAG | 64.5°C | 300 bp |
| Ex2R | GGCGCCACCGCTGCCCACGGAGATG | | |
| Ex2G | GCGCGGAAAACAGGGGTGGC | 64.5°C | 400 bp |
| Ex2H | GCTGCGCCGCCTTGTCCTTG | | |
| Ex3aF | GGGGCTGTGAGCGCTCCAGTACAGCC | 68.1°C | 800 bp |
| Ex3aR | CCTGGCTGGTGGCCCGGGTGTGCGCCAA | | |
| Ex3bF | TGGCGGTGCCGGTCCTGGTGAAAGA | 55°C | 458 bp |
| Ex3bR | CCAGGTTGTTAAGAAAAGTCG | | |

**Table 1**. NKX2-1 Primers and annealing temperatures

| Exon | Sequence | Tm | Lenght bp |
|---|---|---|---|
| Ex3F | CATAGCTAATCCCCACCCAAAC | 58°C | 259 bp |
| Ex3R | GCCTGCGGTGAATTTCG | | |
| Ex4F | ATTGGGTAATTCTTTGGGATTC | 59°C | 314 bp |
| Ex4R | CCAGGCCTTTCTTGTCTCTT | | |
| Ex5F | AGGGGTGTCAAAAAGGCGACTG | 61°C | 241 bp |
| Ex5R | TGGGTATGCTGAAGGGGAGGTG | | |
| Ex6F | TCTCCCTCTCCCCCACTG | 58°C | 255 bp |
| Ex6R | GCAGAGCCCCTACAAAGTCC | | |
| Ex7F | GAGCATGAATGATAGGTCCC | 58°C | 304 bp |
| Ex7R | CACAGGCTCATTTGGAGAAT | | |
| Ex8F | GTCTCTGTGCGCTGACTTCT | 59°C | 232 bp |
| Ex8R | CACACCTTCCGCCTGAC | | |
| Ex9F | CCTCCCCGCCATCTCACACC | 61°C | 290 bp |
| Ex9R | TCCCACCCGCCGCCATAG | | |
| Ex10F | GCCCCCATGGTCCAACTGAC | 61°C | 220 bp |
| Ex10R | TGCCTCTGCTCCTTGTGTCCAC | | |

**Table 2**. PAX8 Primers and annealing temperatures

| Exon | Sequence | Tm | Lenght bp |
|---|---|---|---|
| Fr1F | CACCATGCAGGGAAGCTG | 59,1°C | 250 bp |
| Fr1R | CCTCTGGCTTGAAGGCG | | |
| Fr2F | AGCTCTCTGCCCGCCTG | 55,5°C | 300 bp |
| Fr2R | AGTTTCTTGGGGACGAAAGC | | |
| Fr3F | AGGTCAAGCCGCTCTTAC | 58°C | 218 bp |
| Fr3R | TTCGGGGGCCGACAGGTA | | |
| Fr4F | AGGCGCAGGTCTATGAGC | 64,5°C | 430 bp |
| Fr4R | CCATCGCGCACCAGCACT | | |
| Fr5F | GCGGCAGGACCAGACTCT | 64,7°C | 300 bp |
| Fr5R | TGTTGGCGGCGGCAGTGG | | |

**Table 3**. NKX2-5 Primers and annealing temperatures

| Exon | Sequence | Tm | Length bp |
|---|---|---|---|
| J 1F | AGAACACAATTCCCAGAGGG | 60.9 °C | 266 bp |
| J 1R | AGAGCGGATGCCAGCAG | | |
| J 2F | ATTCCCCTTACCCATTGAGC | 62 °C | 253 bp |
| J 2R | GCTTACACCCCACAGCACTC | | |
| J 3aF | TTTTGAGGGAGCACACCATT | 56.7 °C | 156 bp |
| J 3aR | ATTCCGATGAAGGAGGCTGT | | |
| J 4aF | CCGGGGCTCTGTCTTTACTC | 60.9 °C | 222 bp |
| J 4aR. | GCTTCAGGTGAGGGAGATGA | | |
| J 5F | TGATTGAGACTTTGGGGAGG | 60.9 °C | 272 bp |
| J 5R | CTCTCTAGCCACGGCGAC | | |
| J 6aF | TGGACCTGCACAGAATTGTC | 60.9 °C | 163 bp |
| J 6aR | ATAGGCGGGGTGATAGACCT | | |
| J 7aF | CTTCTTGCTCTGGGCTGCAT | 60.9 °C | 207 bp |
| J 7aR | CGAGCCACAGGAAGCATCTAT | | |
| J 8F | GAGGGTGGGAGGATGTCTG | 62.7 °C | 266 bp |
| J 8R | AGGGAAGTTCAGGCATTGAG | | |
| J 9F | GTAGCCCAGGCTTGTCTCTG | 60.9 °C | 266 bp |
| J 9R | TCCCTAGGAGTGCAGACCAC | | |
| J 10aF | GGACAGGACTAGATGCTGTGTTTG | 60.9 °C | 223 bp |
| J 10R | TTGGAAGGAAAGAGCCTGAG | | |
| J 11F | CTCGGCACCGTACTCGG | 60.9 °C | 310 bp |
| J 11R | GCCCAGCACCTTATACCTATG | | |

**Table 4.** DNAJC17 Primers and annealing temperatures

*3.5 Mutational Screening*

3.5.1 SSCP analysis

PCR products of *PAX8* and *NKX2-5* gene, were diluted 1:1 in a denaturing solution (99% formamide, 1 % xylene-cyanol solution and bromophenol blue), denatured at 95 °C for 5 min and thereafter diretly placed on ice to prevent reanniling of the single stranded product. PCR products were loaded in non – denaturing gels 12% Polyacrylamide (GeneGel Excel 12.5/24 Kit, GE Healthcare Bio-Sciences AB, Sweden) using the GenePhor Electrophoresis Unit (GE Healthcare Bio-Sciences AB, Sweden).
The gels were run at 8 °C and a constant Voltage of 650 V, Current of 30 mA, Power of 15 W, for 3 hours (until bromophenol blue reaches the anode buffer strip).

SSCP bands were detected with the PlusOne DNA Silver Staining Kit (GE Healthcare Bio-Sciences AB, Sweden) contained Fixing Solution, Staining Solution, Sodium Carbonate Solution, Formaldehyde 37%, Sodium Thiosulphate 2% and Stopping Solution. Then, the gels were air dried. Samples showing an abnormal mobility pattern within the matrix when compared with the wild-type control, were submitted to direct sequencing.

### 3.5.2 High Resolution DNA Melting

Mutation screening of exons 1 to 10 covering the *DNAJC17* coding region was performed by High Resolution Melt Analysis. Primers for HRM curve analysis were similarly designed to flank the coding regions and to be annealed at 60°C using Primer Express software to calculate the "Tm" (Applied Biosystems, Foster City, CA). Real-time PCR and subsequent high-resolution melt analysis were performed in 20-µl mixture on StepOne Plus (Applied Biosystems, Foster City, CA USA). The MeltDoctor HRM Master Mix (Applied Biosystems, Foster City, CA USA) contains magnesium chloride, dNTPs, MeltDoctor™ HRM Dye and AmpliTaq Gold 360 DNA Polymerase at a 2x concentration. The Melt Doctor™ HRM dye is a stabilized form of the SYTO®9 dye, a next-generation ds DNA-binding dye developed by Molecular Probes that delivers sharp, clean melt profiles. Simplest to use is MeltDoctor™ HRM Master Mix; it requires only the addition of template DNA and a PCR primer pair before starting the PCR. We used 200 nM of each PCR primer and 20 ng of genomic DNA in 20 µL PCR reactions.
PCR conditions and primer sequences are shown in table 4.
Melting-curve data were generated by increasing the temperature from 72 °C to 95 °C at 0.3 °C/ and recording fluorescence in the green channel. HRM curve analysis was performed using StepOne Software (Version 2.3). The software was used to calculate the derivative of the intensity of fluorescence at different temperatures (dF/dT). The reference strains were included in each run to facilitate inter-run comparisons. All samples were tested in duplicate to ensure reproducibility of the melt curves. The HRM curves were derived by first selecting two normalization regions, one occurring prior to the melting of the double stranded product and one following complete separation of the two strands. If a sample showed an aberrant melting curve pattern, the PCR product was purified with ExoSAP and sequenced for both forward and reverse strands with ABI Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA USA) according to the standard protocol. All mutations were also verified on PCR products directly using genomic DNA as a template.

### 3.5.3 Direct DNA sequencing

The three exons of the *NKX2-1* gene were direct amplified by polymerase chain reaction (PCR) as previously described for all Brazilian family's members. PCR products were purified with Antarctic Phosphatase - Exonuclease I (NewEngland BioLabs, Ipswich, MA) at 37 C° for 15 minutes and 80 C° for 15 minutes for the enzyme activation and inactivation respectively and the products were bidirectionally sequenced with a 377 ABI sequencer (Applied Biosystems, Foster City, CA). The genes encoding *PAX8, NKX2-5* were also amplified and sequenced as previously described. To exclude the presence of polymorphysms, 100 normal subjects were also screened as controls.
All the samples resulted abnormal to either SSCP or HRM PCR were also re-amplified and sequenced with the same technique.

## *3.6 Mutagenesis*

The mouse Nkx2-1 gene from the p3XFLAG-CMV-10-NKX2-1 vector was mutagenized by QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's procedure, using as template in a PCR the sense primer 5'-CCGCCGGAAGCGCGGGTGCTCTTCT-3' and the antisense primer 5'-GAGAAGAGCACCCGCGCTTCCGGC-3' for the NKX2-1 c.493delC.
For the PAX8 c.T1009G, the mouse Pax8 gene from the p3XFLAG-CMV-pax8 vector was mutagenized using the same protocol. It was used as template in a PCR the sense primer 5' AGCAAGTCGGCGCCGGGGTCCCG 3'- and the antisense primer 5' CGGGACCCCGGCGCCGACTTGCT 3'
The presence of both mutations in the obtained vectors was confirmed by direct sequencing.

## *3.7 Cell culture and in vitro functional assays*

HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2mM L-glutamine, 4.5 g/L D-glucose, 50 U/mL penicillin, 50 µg/mL streptomycin, and 10% fetal bovine serum under humidified 5% carbon dioxide/95% air at 37C° and grown in 24-well plates to 70%–80% confluence (i.e., ˜5 x $10^4$ cells per well). HeLa cells were transfected with the reporter plasmid (0.1 µg of pTg-Luc, 0.3µg of SP-C-Luc 0.32) plus 3 ng of CMV-Ren as an internal control vector, and different doses of effector plasmids. HEK293 cells were transfected with 0.1 µg of the reporter pTg-Luc plasmid and 1.5 ng of CMV Ren internal control vector plus different doses of

effector plasmids. In each set of transfection experiments, the total amount of transfected DNA was normalized using an empty vector. Transfections were performed with the TurboFect in vitroTransfection Reagent (Fermentas Life Sciences, Pittsburgh, PA), using a DNA/Turbofect ratio of 1:2 in all experiments. Cells were harvested after 36–48 hours and analyzed sequentially for firefly and Renilla luciferase activities (Dual-Luciferase Reporter Assay System, Promega, Madison, WI). The ratios between the measured firefly and Renilla luciferase activities were expressed relative to the ratios obtained in cells transfected with the reporter and an empty expression vector (CMV-Flag) only. Transfection experiments were performed in duplicate and repeated at least three times. Data are shown as mean ±SD. Statistical analysis was performed using Student's t-test.

*3.8 Western blot*

For western blot, cells (5 x $10^6$) were plated on 100-mmdiameter culture dish 24 h before transfection. HEK293 cells were transiently transfected with 10 ng of either wild-type (WT)-Nkx2-1 or c.493delC-Nkx2-1 plasmids using the FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. After 48 hours, cells were scraped in phosphate-buffered saline (pH 7.4) and centrifuged at 1792 g for 5 minutes at 4C°. Pellets were frozen and stored at -80C° until used to prepare nuclear or total extracts. To obtain nuclear extracts, the frozen pellets of the transfected HEK293 cells were resuspended in low-salt buffer (10mM HEPES, pH 7.9; 10mM KCl; 1.5mM MgCl2; 0.1mM EGTA [pH 7]; 0.5mM dithiothreitol [DTT]; pepstatin, 4mg/mL; 2mM benzamidine; aprotinin, 20 mg/mL; 10mM leupeptin; and 1mM phenylmethylsulfonylfluoride) and centrifuged for 3 minutes at 4 °C at 2000 rpm. The pellets were resuspended in low-salt buffer and passed through a 25-gauge needle for 15 times. Nuclei were pelleted by centrifugation at 5000 rpm for 3 minutes at 4 °C and resuspended in 100 µL of extraction buffer (10mM HEPES, pH 7.9; 0.4 M NaCl; 1.5mM MgCl2; 0.1mM EGTA [pH 7]; glycerol 5%; 0.5 mM DTT; pepstatin, 4mg/mL; 2 mM benzamidine; aprotinin, 20 mg/mL; 10 mM leupeptin; and 1mM phenylmethylsulfonylfluoride) and incubated for 30 minutes at 4 °C. After centrifugation at 12,000 rpm for 30 minutes at 4 °C, the supernatant was quantified by BioRad Assay.
Total extracts of transfected HEK293 cells were prepared as follows: frozen pellets of the transfected HEK293 cells were resuspended in 1mL Busslinger Lysis Buffer plus proteases inhibitors (20mM TRIS, pH 7.9; 120mM KCl; 5mM MgCl2; 0.2mM EDTA; 0.2% NP-40; 10% glycerol; DTT 100x; NaF40x; β- glycerophosphate 40x; and NaV [1000x]) and chilled on ice for 15 minutes. After centrifugation for 10 minutes at 12,000 rpm, the supernatant was frozen at -80 °C. Prior to use, the protein content of each sample was quantified by BioRadAssay. Ten micrograms and 40 µg each of nuclear and total extracts

were boiled in Laemmli buffer and resolved on a 12% SDS PAGE gel. The gel was blotted on Immobilon P (MilliporeCorp., Bedford, MA) for 90 minutes at a constant current of 100 mA. Immunodetection of NKX2-1 was performed by using a monoclonal anti-FLAG antibody (M2; Sigma, St.Louis, MO) diluted 1:3000 in Tris-buffered saline containing 5% nonfat milk (Bio-Rad Laboratories, Inc., Richmond, CA), and the filter was treated with a 1:3000 dilution of goat antimouse IgG conjugated to horse radish peroxidase (Bio-Rad).

### *3.9 Electrophoretic mobility shift assay*

We evaluated the binding of Nkx2-1 to DNA in the presence of Nkx-c.493delC by electrophoretic mobility shift assay (EMSA) using double-stranded end-labeled oligonucleotides C for the Tg promoter as described (Santisteban P, Acebron A et al. 1992), and double-stranded end-labeled oligonucleotide C2 for the surfactant protein C (SP-C) promoter.
Antisense oligonucleotides were labeled with a T4 polynucleotide kinase (New England Biolabs, Boston, MA) and radioactive [-32P] ATP. Double-stranded oligonucleotides were purified by passing them through nick columns containing Sephadex G-50DNA grade resin (Amersham-Pharmacia). Nuclear extracts were prepared as previously described from transfected HeLa cells. EMSAs were performed with 30-µl reaction mixtures at room temperature at a final concentration of 20 mM Tris-HCl, pH 7.5, 75 mM KCl, 1 mM dithiothreitol, 10% glycerol, and 1 mg/ml poly (dI-dC). Binding reaction mixtures containing 1 µg of nuclear extracts and 10 fmol of the probe were equilibrated for 30 min at room temperature and separated by electrophoresis through a 6% polyacrylamide gel containing 0.5x Tris-borate-EDTA. Binding specificity was assessed by competition with a 100-fold molar excess (or as indicated) of cold competitors incubated with proteins 15 min before the probe was added.

### *3.10 Review of the literature*

We performed a systematic review of the literature by a MEDLINE search using the key words ‘congenital hypothyroidism’, ‘benign hereditary chorea’, ‘thyroid transcription factor 1’, ‘NKX2-1’, ‘TITF1’, ‘TTF1’ and ‘mutation’. Inclusion criteria for the analysis were (i) patients with documented mutational analysis and (ii) individual clinical data on thyroid, lung and neurologic disorders. All patients with NKX2-1 gene defects reported in the literature were reviewed. Clinical features (age at diagnosis of CH, IRDS, CILD and BHC), data on laboratory and imaging investigations (result of neonatal

screening, TSH, T4, T3, thyroid ultrasound or scintigraphy, cerebral MRI) were entered in a database. All patients with isolated BHC without documented thyroid function were excluded.

# 4. RESULTS

## *4.1 Mutational screening of genes*

### 4.1.1 Mutational screening of *NKX2-1*

Single Strand Chain Polymorphism (SSCP) was used to screen for mutations in the *NKX2-1* coding sequence using specific primer-pairs (Table 1) in 48 patients affected by CH (Table 5). No abnormal bands were detected in any of the studied patients.

Direct sequencing was used as first screening method in the patients of the Brazilian family showing the clinical features of the BTL syndrome. Sequencing of the *NKX2-1* coding region of the proband's DNA in the Brazilian family revealed a new monoallelic deletion of the cytosine at position 834 (accession no. NM_003317.3) (Fig. 8). The mutation is responsible for a frameshift that produces an abnormal protein from amino acid 165. The last amino acid of the nuclear localization signal (NLS), the whole homeodomain, and the carboxy-terminus of NKX2-1 are all missing in the mutant protein, which has a premature stop codon at position 196 (p.Arg165Glyfs*32). The mutant protein has an approximate molecular weight of 20 kDa. The same mutation (TRANSNP_1331899076), never described before, was present in all the affected members of the family but in none of the unaffected members of the family and in 100 normal controls.

### 4.1.2 Mutational screening of *PAX8*

SSCP was used as first approach to screen for mutations the entire *PAX8* coding sequence using the specific primer-pairs (Table 2) in 48 patients affected by CH (Table 5).

An abnormal band was observed in the migration pattern of exon 9 of *PAX8* gene in a 12yo girl affected by thyroid ectopy (sublingual thyroid). She was diagnosed at birth as congenital hypothyroidism and received substitutive treatment since she was 17 days old. No family history of thyroid disease has been reported. The mother of the patient has been adopted.

Sequencing of the abnormal sample identified by *SSCP* revealed an heterozygous mutation at position 1009 of the *PAX8* (c.T1009G, reverse strand). The mutation is responsible for a change of the first nucleotide of the triplet encoding for serine at position 337 (Ser337Ala). The mutation was not present in 100 DNA samples of normal controls, but sequencing of the family of the patient demonstrated that it was inherited from the mother (Fig. 9).

| | | Mutational screening | |
|---|---|---|---|
| **CH Patients** | **N. of cases (76)** | **SSCP** *(NKX2-1, NKX2-5, PAX8)* | **HRM** *(DNAJC17)* |
| Athyreosis | 17 | 7 | 10 |
| Ectopia | 31 | 17 | 14 |
| Hypoplastic | 4 | / | 4 |
| Hemiagenesis | 19 | 19 | 19 |
| Normal thyroid | 5 | 5 | / |

**Table 5**. Resume of N. of cases with CH and mutational screening techniques used.

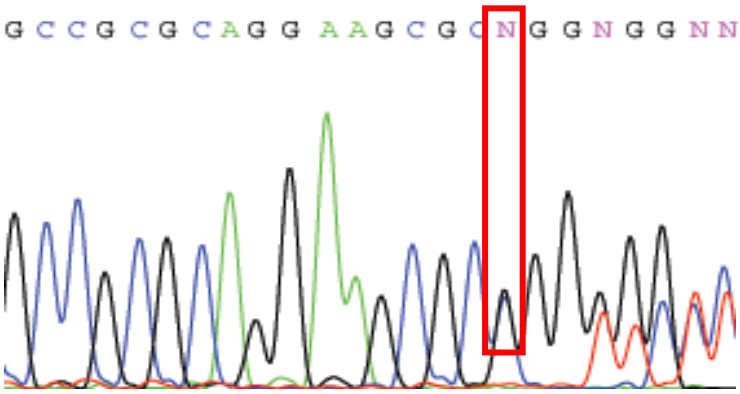


**Fig. 8**. Electopherogram showing a *NKX2-1* mutation. The c.493delC mutant produces a frameshift with a premature termination of the protein at the 196 aminoacid (p.Arg165Glyfs*32). The mutation is present in all affected members of the family.

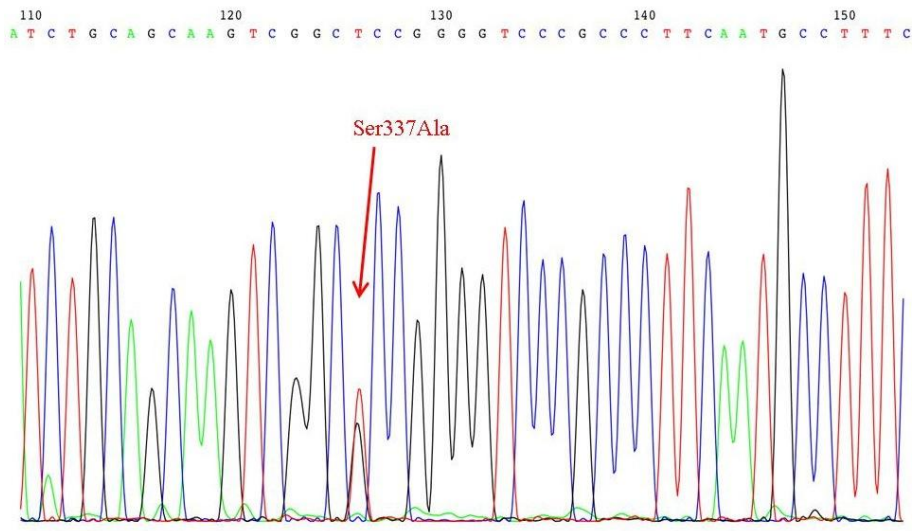


**Fig. 9** Electropherogram of *PAX8* exon 9. We identified a variant c.1009T>G (p.Ser337Ala) in a patient with thyroid *ectopia***.**

### 4.1.3 Mutational screening of *NKX 2-5*

Single Strand Chain Polymorphism (SSCP) was used to screen for mutations in the *NKX2-5* coding sequence using specific primer-pairs (Table 3) in 48 patients affected by CH (Table 5). No abnormal bands were detected in any of the studied patients.

### 4.1.4 Mutational screening of *DNAJC17*

High Resolution DNA Melting Analysis (HRM) was used as first approach to screen for mutations in the *DNAJC17* coding sequence using specific primer-pairs. Forty-five patients with permanent CH (19 with emiagenesis, 9 with athyreosis, 4 with hypoplasia, 13 with ectopia) and 8 controls were included in the study. Analysis of the HRM data shows normalised plots and difference plots of each mutant compared to wild-type controls for amplicons for exons 1 to 12 of *DNAJC17* . The majority of the melt profiles were clearly distinguishable on difference plots from wild-type by amplitude and/or by shape. In particular, among all the samples studied we

observed the presence of aberrant melt profiles in some cases with changes in melting curve shape in *DNAJC17* exons 5 (Fig.10).
Direct sequencing of samples resulted abnormal to *HRM analysis* (ex. 5) revealed the presence of a sequencing variant only in exon 5. In particular, we identified a substitution of adenine to citosine in exon 5 (c.350A>C) of *DNAJC17* in a patient affected by athyreosis.

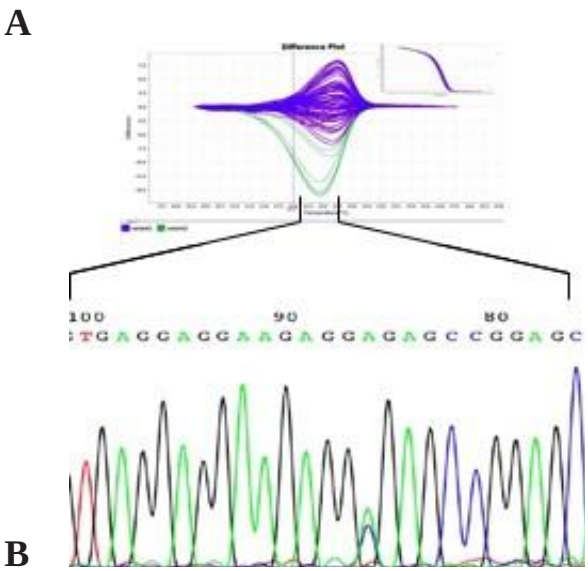


**Fig. 10** HRM (A) and electropherogram (B) of *DNAJC17* exon 9. We did not identify any base change in a patient with *ectopia*.

*4.2 SNPs Data Base*

The identified *NKX2-1* (c.493delC), *DNAJC17* (c.350A>C) and *PAX8* (c.1009T>G) variants were queried into the NHLBI GO Exome Sequencing Project (ESP) database, to estabilish if they were new mutation or previously reported polymorphisms. This database includes some of the largest well-phenotyped populations in the United States, representing more than 200,000 individuals.

The c.350A>C-*DNAJC17* mutant was present in the database as polymorphism. The nucleotide at position 350 of *DNAJC17* was reported as adenine in 99% of the alleles, while the citosine has a frequency of 1%. The

prevalence of cytosine is higher in European population, where it represents the 3% of the cases.

Also the c.1009T>G-*PAX8* on Ensemble Genome Browser demonstrated that this variant is a known SNP (rs199939219) (Fig. 11), while the search of the c.493delC-*NKX2-1* produced no results, indicating that this is a de novo mutation associated with the brain–thyroid–lung syndrome (BTLS) (Fig. 12).

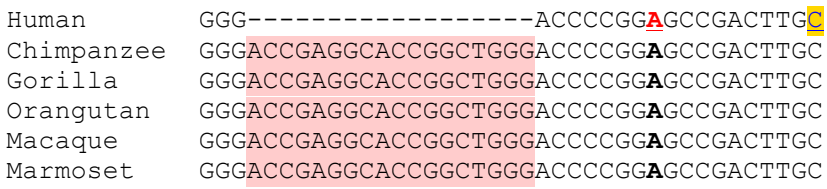


**Fig. 11**. Philogenetic context of rs199939219 SNP (c.1009T>G PAX8). The alignment of 6 primates shown variants (including SNPs and indels) imported from dbSNP. The A/C (ancestral of T/G) is conserved in species.

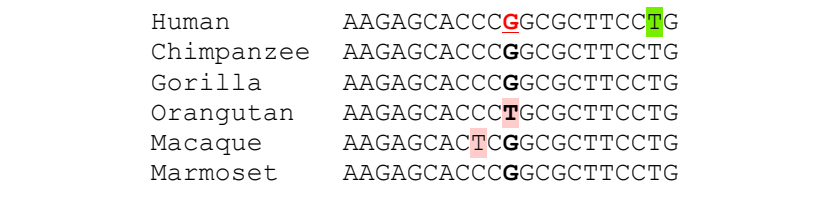


**Fig. 12**. NKX2-1 Sequence conservation (ancestral) via cross-species alignments.

*4.3 PAX8 Functional characterization*

As indicated, the c.1009T>G was present as rare polymorphism in the tested databases. Since no functional data were available for this variant and in order to exclude a possible role of this polymorphism ass co-factor in the pathogenesis of thyroid dysgenesis, we performed functional studies on the c.1009T>G-PAX8. To this aim, the c.1009T>G-PAX8 was inserted by site directed mutagenesis in an expression vector and transfection experiments have been performed to test the functional effect of the mutation. The mutant protein had the same activity of the WT-PAX8, did not produced a dominant negative effect and the activity in presence of NKX2-1, TAZ or P300 is similar to the activity measured for the wild type. This data confirmed that the c.1009T>G – PAX8 has no functional effect on PAX8 function anc can be considered as a simple polymorphism with no clinical relevance.

*4.4 NKX2-1 functional characterization*

4.4.1 Nuclear localization

Since the c.493delC mutation occurs in the codon encoding for the last amino acid of the NLS of NKX2-1, we tested the effect on the nuclear localization. To this aim, HeLa cells were transfected with either the WT-Nkx2-1 or the c.493delC-Nkx2-1. After 24 hours, both nuclear and cytoplasmatic proteins were extracted from transfected cells and Western blot results are shown in Fig. 13. As expected, WT-NKX2-1 was synthesized and migrated into the nucleus, disappearing from the cytosolic extracts. Despite the alteration of the nuclear localization signal, thec.493delC was able to migrate into the nucleus. These results indicate that the p.Arg165Glyfs*32 was synthesized and was capable of migrating into the nucleus.

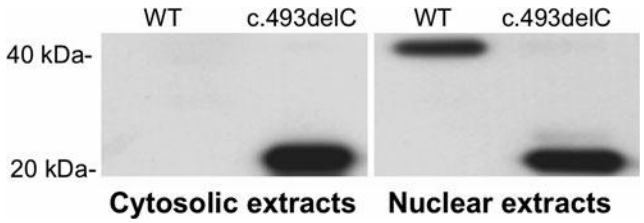


**Fig. 13**. Nuclear Localization. Western blot analysis with the anti-FLAG antibodies of cytosolic and nuclear extract of the cells transfected with both wild-type (WT) and c.493delC (p.Arg165Glyfs*32) cloned into the p3XFLAG-CMV-10 plasmid. The c.493delC is normally synthesized and able to migrate into the nucleus.

4.4.2 DNA binding capacity

DNA binding properties of the c.493delC mutant were also analyzed by EMSA using a short DNA stretch correspondingto the NKX2-1 binding site within the Tg promoter (oligo C) and the SP-C promoter (oligo C2). The results are shown in Fig. 14. A retarded band could be detected with the WT, while the c.493delC NKX2-1 was, as expected, unable to bind to DNA because of the absence of the homeodomain. Moreover, the addition of the same or double amount of c.493delC extracts did not reduce the binding of WT-NKX2-1. This indicates that c.493delC-NKX2-1 was unable to physically interact and modulate WT-NKX2-1 binding to DNA. The specificity of all the observed complexes was demonstrated in competition experiments adding a 100-fold excess of cold oligos.

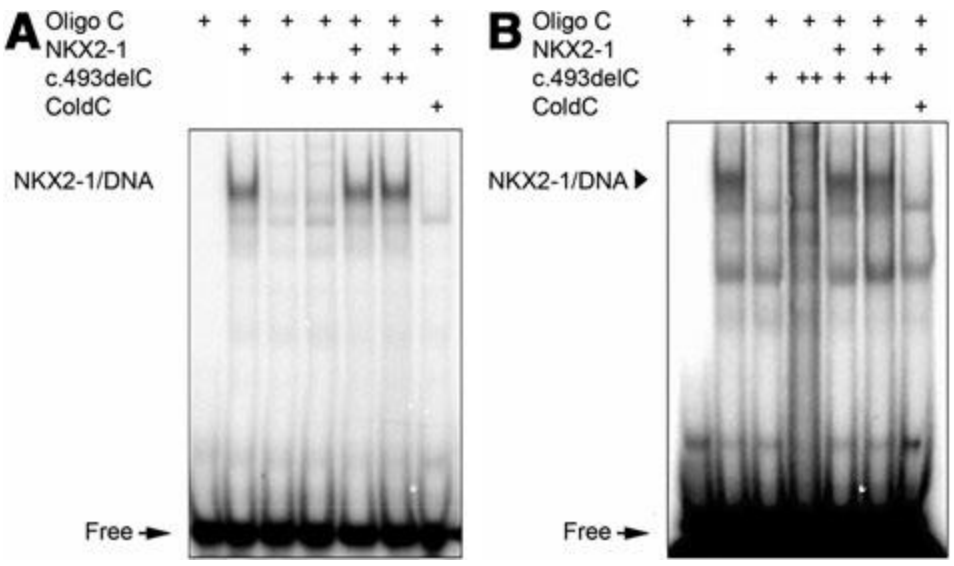


**Fig. 14**. DNA binding capability of the c.493delC mutant (p.Arg165Glyfs*32). Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts of HEK293cells transfected with WT and c.493delC constructs on the thyroglobulin (Tg) promoter (A) and surfactant protein C (SP-C) promoter (B). Arrowheads indicate the bound NKX2-1 to the oligos. The co-transfection of the WT-Nkx2-1 with the same (+/+) or double (+/++) amount of the mutant c.493delC does not reduce the binding of the WT. Extracts were also incubated with a large excess (100-fold) of cold oligos as a competitor that produced a significant displacement of the bound labeled probe.

4.4.3 Transactivation capacity of mutated and WT NKX2-1 proteins

The transcriptional properties of both WT- and c.493delC NKX2-1 were investigated by co-transfection assays as previously described. As expected, the c.493delC mutant was unable to activate both the Tg and the SP-C promoters. Thereafter co-transfection experiments were performed using both WT and the c.493delC mutant on the same promoters. Interestingly we were able to demonstrate a dose dependent dominant negative effect of the c.493delC mutant on the WT-NKX2-1 when used with the Tg promoter (Fig.15A), but no dominant negative effect could be demonstrated on the SP-C promoter (Fig. 15B), indicating that the c.493delC mutant produced a dominant-negative effect on the wild-type NKX2-1 in a promoter-specific manner. To further understand the dominant-negative action and investigate the c.493delC effects, co-transfection experiments were performed with PAX8, P300, and TAZ on the Tg promoter.

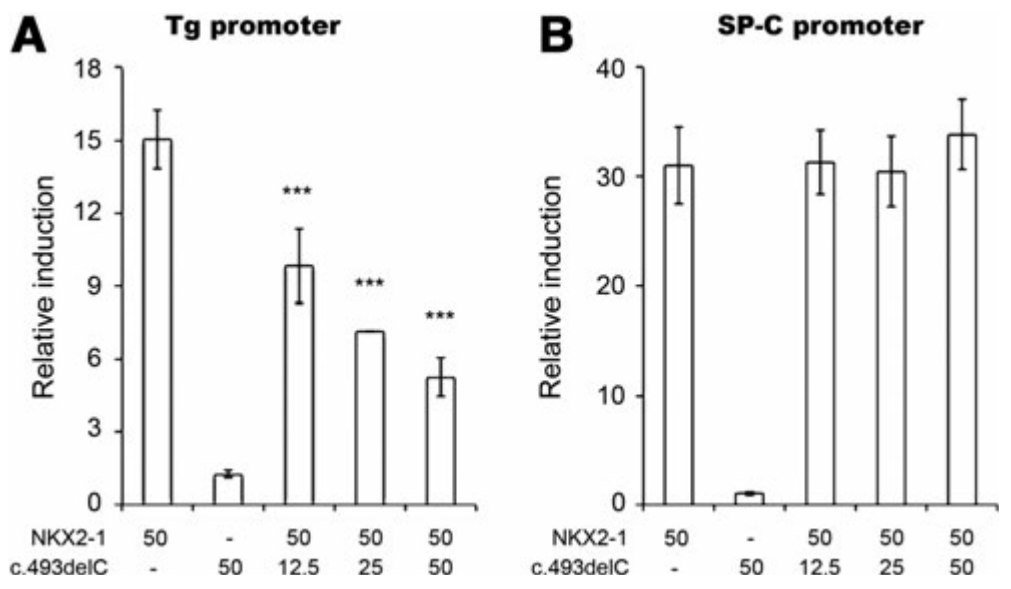


**Fig. 15**. Transcriptional activation of Tg and the SP-C promoters.(A) Effects of transfection of WT-NKX2-1 and c.493delC-NKX2-1 on the Tg promoter. Addition of increasing amounts (12.5, 25, 50 ng) of c.493delC to a constant amount (50 ng) of the WT produced a significant decrease in the promoter activity (***$p < 0.001$). (B) Effects of transfection of WT-Nkx2-1 and c.493delC-Nkx2-1 on the SP-C promoter. Contrary to what was observed for Tg, addition of increasing amounts (12.5, 25, 50 ng) of c.493delC to a constant amount (50 ng) of the WT did not reveal a dominant negative effect.

PAX8 (Paired Box Gene 8) is a transcription factor expressed in the thyroid but not in the lung. It synergizes with NKX2-1 to stimulate Tg and thyroperoxidase promoter activities (Di Palma, Nitsch et al. 2003). P300 (E1A-Binding Protein), a general transcriptional coactivator, plays a pivotal role in the activity of many transcription factors, either by bridging sequence-specific DNA binding factors with elements of the basal transcriptional machinery, such as transcription factor IIB and polymerase 2, or by its intrinsic histone acetyl transferase activity. NKX2-1, PAX8, and P300 physically interact with each other (Di Palma, Nitsch et al. 2003), and the functional synergism may involve the direct cooperation of all three factors. The addition of P300 increases the transactivation properties of PAX8, NKX2-1, and both factors together. TAZ (transcriptional co-activator with a PDZ-binding motif, also known as WWTR1) is also present in thyroid tissue and in differentiated thyroid cell lines, thus acting as a potent co-activator for both PAX8 and NKX2-1 (Park, Whitsett et al. 2004). As previously shown (Di Palma, Nitsch et al. 2003), we were able to demonstrate that PAX8 synergized with the WT protein and increased Tg promoter activity. The cotransfection with the c.493delC did not modify the PAX8 basal activity, and the dominant negative effect was also present when both WT and c.493delC proteins were transfected with PAX8 (Fig. 16).

To further study the interplay of P300, PAX8, and both WT and c.493delC proteins, cotransfection experiments were performed in HEK293 cells, since these cells lack the endogenous P300. Again, if the c.493delC was added to the cells, there was no increase in the activation produced by PAX8 and/or P300, suggesting that this truncated protein did not interfere with the complex. Finally, when all four factors were present (WT, c.493delC, PAX8 and P300), a slight decrease in the activity could be observed compared with the multiprotein complex of NKX2-1/PAX8/P300 (Fig. 17). These results indicate that the dominant negative effect can also be observed when a multifactorial complex is present, and PAX8 or P300 are unable to rescue the effect of the mutation. Similar results were obtained when the coactivator TAZ was used in HeLa cells.

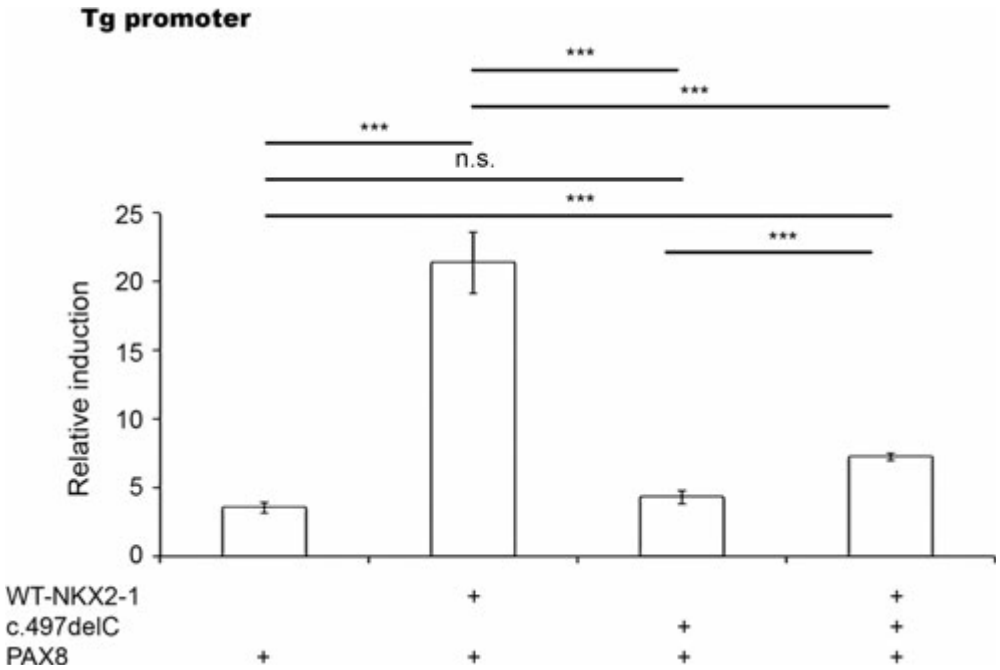


**Fig. 16**. Synergism with PAX8. Transcription activation of the Tg promoter by WT-NKX2-1 and c.493delC-NKX2-1 in the interaction with PAX8. HeLa cells have been transfected with 50 ng of each plasmid and 100 ng of the reporter. The addition of WT produced a significant (***p < 0.001) increase in the PAX8 activity, while the addition of the c.493delC hadno effect. If both WT-Nkx2-1 and c.493delC-Nkx2-1 were transfected, the dominant negative effect of the mutant on the WT protein was still present. The presence of PAX8 did not rescue the dominant negative effect on the Tg promoter.

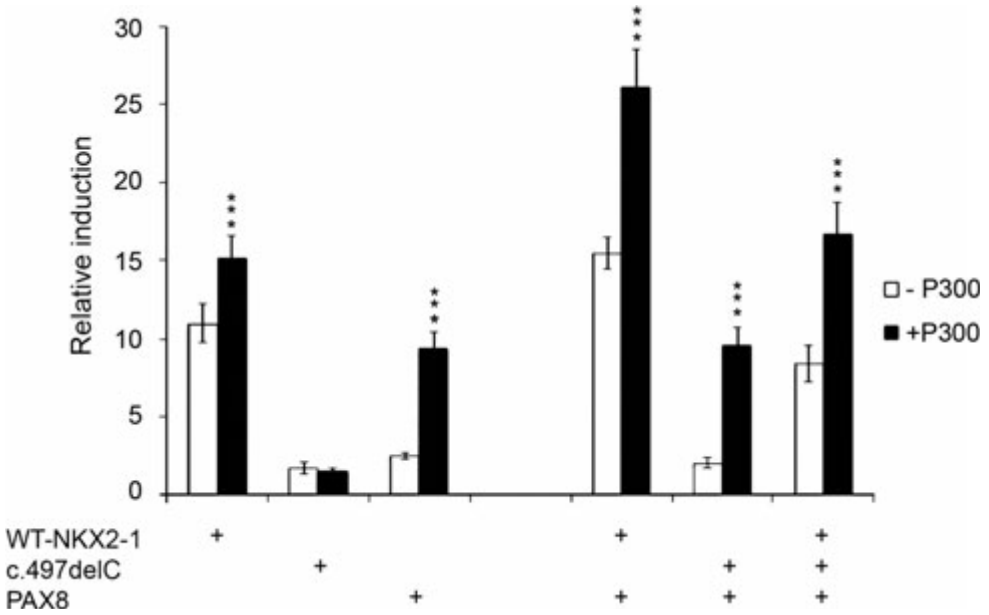


**Fig. 17**. Effect of P300. HEK293 cells were co-transfected with WT-Nkx2-1 (50 ng), c.493delC-Nkx2-1 (50 ng), PAX8 (50 ng), and P300 (300 ng) expression vectors, and the pTg-Luc reporter (100 ng) in the indicated combinations. The addition of P300 significantly increased the activity of the Tg promoter in response to WT-NKX2-1 and PAX8, but had no effects on the activity of c.493delC. The cooperation between WT, PAX8, and P300 produced an additional increase in the activity, while the activation of the complex c.493delC/PAX8 did not differ from the activation observed when PAX8 was co-transfected with P300 alone. Finally, when all the factors were present, there was a reduction of the activation compared with the WT+ PAX8 complex, as a consequence of the dominant negative effect of the c.493delC mutant on the WT. Significant differences between the samples with and without P300 are indicated: ***$p < 0.001$.

# 5. DISCUSSION

The development of the thyroid gland is a complex process of organ induction in the pharyngeal endoderm, early budding of the organ anlagen, tissue relocalisation and growth. Defects at any of these steps of the thyroid organogenesis can lead to thyroid dysgenesis (TD). With this term, several alterations of the gland are indicated, including the complete absence of thyroid tissue (athyreosis), the presence of an ectopic organ or hypoplasia of the gland.
The molecular basis of thyroid development have only recently been studied with the identification of several genes playing a crucial role in the thyroid organogenesis.
Comparative and functional studies in different animal models have provided interesting hypotheses for the mechanisms by which the developing thyroid anlagen is correctly positioned in the anterior neck region (De Felice M and Di Lauro R 2004). Alterations of the thyroid morphogenesis, at least in some cases, have been associated with disturbances in the function of genes that regulate several aspects of thyroid development, suggesting that TD can be a heritable genetic disease. However, in the majority of patients, the search for mutations in the known genes produced no results.
In order to further identify genetic defects in thyroid dysgenesis we performed a sequencing study of all coding exons of *NKX2-1* in a family from Curitiba, affected by Brain-Lung-Thyroid Syndrome. We also screened 76 patients with persistent CH for known and new candidate genes (*NKX2-1, NKX2-5, PAX8, DNAJC17*) by SSCP or HRM.
Mutations in NKX2-1 have been demonstrated to be responsible for several cases of BTLS, and in our study we describe a novel mutation in the gene encoding for NKX2-1 in a family with the clinical manifestations of benign hereditary chorea, hypothyroidism, and respiratory distress. The mutation c.493delC results in a truncated protein of 196 amino acids. The mutant protein contains a frameshift and differs from the WT beginning at position 165, which corresponds to the last amino acid of the NLS. Transfection experiments with the mutant protein demonstrate that the c.493delC protein is synthesized and translocates into the nucleus, but it is unable to bind DNA since it completely lacks the homeodomain. The c.493delC mutant was responsible for a clear dose-dependent dominant negative effect on the WT NKX2-1 when tested on the Tg promoter, whereas when functional assays were performed on the lung SP-C promoter, the c.493delC mutant showed no dominant negative effect.
Several mutations in NKX2-1 have been reported (Table 7), and in the majority of cases haploinsufficiency has been considered to be responsible for the phenotype, as also suggested by the finding of motor abnormalities and mild hyperthyrotropinemia in the Nkx2-1 heterozygous knockout mice (Pohlenz, Dumitrescu et al. 2002). Only a few mutations (Table 7) produce a dominant

negative effect on the WT NKX2-1 (Carre, Szinnai et al. 2009) (Guillot, Carre et al. 2010), and there is only one case among them describing a promoter-specific dominant negative effect (Moya, Perez de Nanclares et al. 2006). In this last case, contrary to what was observed for the c.493delC, the mutant protein contained the homeodomain and could bind to DNA. The authors suggested that the possible interference of the mutant NKX2-1 (p.P275fs*74) with the cooperative interaction between NKX2-1 and PAX8 or NKX2-1 and other cofactors on the Tg promoter is the cause of the promoter-specific dominant negative effect. Since our mutant did not bind to DNA, it is probable that the amino terminus of NKX2-1 binds to other factors that are relevant for its activity on the Tg promoter, thus excluding them from the tissue-specific transcriptional regulation is achieved by the combined interaction among transcription factors, co-regulators, and other components regulating basal transcription. In the thyroid, NKX2-1 physically interacts with both PAX8 (Di Palma, Nitsch et al. 2003) and TAZ (Di Palma T 2009). In addition P300, a ubiquitous transcriptional cofactor, has been demonstrated to play an important role for the NKX2-1/PAX8 synergy (Grasberger, Ringkananont et al. 2005) (De Leo R 2000). PAX8, P300, and TAZ were tested to see whether they could influence the transactivation of NKX2-1 on its target promoters. The presence of these cofactors increased the activation of the Tg and SP-C promoters, but did not change the thyroid-specific dominant negative effect. In addition, no rescue of the NKX2-1/PAX8 synergism was observed, contrary to what was previously reported in the case of a PAX8 mutation (Grasberger, Ringkananont et al. 2005). It has been suggested that NKX2-1 binds to DNA as a dimer (Arnone MI 1995) and that the interaction with PAX8 occurs via the amino terminus of the NKX2-1 protein (Di Palma, Nitsch et al. 2003). Several other factors, most of which not yet defined, may be involved in the regulation of transcription. We believe that the truncated NKX2-1 mutant described here interferes with the activity of WT NKX2-1, decreasing the activity of the multiprotein transcriptional complex. It is likely that PAX8 plays a critical role in modulating this effect in thyroid cells. In lung cells, where PAX8 is absent, the dominant negative effect cannot be observed. Even if HeLa cells do not express PAX8, it has been previously suggested that other proteins with a similar activity may replace the function of PAX8 on the Tg promoter (Moya, Perez de Nanclares et al. 2006). Very recently Silberschmidt and coworkers (Silberschmidt, Rodriguez-Mallon et al. 2011) produced mice lacking one of the two redundant activation domains of Nkx2-1, or having defective phosphorylation of the protein. They demonstrated that each mutant shows a distinct phenotype. They also revealed a level of complexity that could not be predicted by experiments carried out in cultured cells and stressed the discrepancy between the results in the cell models and in those that can be obtained in whole organisms (Silberschmidt, Rodriguez-Mallon et al. 2011). In this context, the major molecular effect of our mutation on the Tg promoter is dominant, while hypothyroidism is only mild (subclinical) in all the affected members of our family. This discrepancy can be the consequence of different

mechanisms of action of the mutation in the different tissues, but also of the complex interactions between other genetic and/or environmental factors that may modulate the phenotype. All the reported NKX2-1 mutations (summarized in Table 7) have variable functional effects, even if the mutations occur in similar regions of the protein. Indeed, the clinical features of the affected families and family members described in the literature are very variable, and there is no correlation between the clinical and the molecular phenotype. Mutations that produce major alterations in the protein structure may be associated with only minor neurological signs (Pohlenz, Dumitrescu et al. 2002) (Provenzano, Veneziano et al. 2008), while primary hypothyroidism has been linked to NKX2-1 mutations in 81% of the cases (25/31) (Table 7).

In conclusion, a novel NKX2-1 mutation in a family presenting the clinical findings of the BTLS is described. The c.493delC mutant migrates into the nucleus but is not able to transactivate the Tg or SP-C promoters. These results indicate that NKX2-1 activity is finely regulated by the interaction of several factors that are still largely unknown. The mechanisms regulating gene expression are different in each tissue. Additional studies are required to better understand the complexities of the genotype–phenotype correlation in the NKX2-1 deficiency syndrome. Both the incomplete penetrance and the variability of the phenotype could be due to the effect of other modifier genes as well as of environmental factors. Furthermore, when the disease is associated with a large chromosomal deletion, we cannot exclude that the loss of other genes is contributing to variability of the phenotype.

In addition to *NKX2-1*, several other genes have been associated to TD. In our study, the *NKX2-1, PAX8* and *NKX2-5* genes were also studied in a cohort of 76 patients with TD. Mutations in these genes have been previously described in cases of athyreosis, ectopia, and hypoplasia (Tables 6-8) with a very high variability in the clinical manifestations.

Dominantly inherited *NKX2-5* mutations are known to cause heart defects, but, despite several mutations have been associated to TD (Dentice, Cordeddu et al. 2006) (Table 8)**,** the involvement of *NKX2-5* in TD in humans is still a matter of debate.

Hermanns and co-authors, reported a girl with TD who is heterozygous for two mutations, each encoding a transcription factor important for the development of the thyroid gland, respectively *NKX2-5* and *PAX8* genes. The *NKX2-5* S265R is located in a highly conserved region of the gene and it is not a common polymorphism. It binds normally to the target sequences because the mutation is located outside the homeobox domain of *NKX2-5,* but presented a reduced transcriptional activity when compared to the WT. Furthermore, S265R-*NKX2-5* exerts a dominant negative effect, thus been likely to have an impact on thyroid organogenesis, even though genetically affected family members did not have TD or heart defects (Hermanns, Grasberger et al. 2011). In addition, in the same patient, it was identified a heterozygous mutation in the *PAX8* gene promoter (-456). The mutated *PAX8* gene promoter showed a reduced ability to activate a reporter gene and does not bind nuclear extracts.

So, it could be contribute to the pathogenesis of TD. It is surprising the observation that a younger sibling of patient, also heterozygous for both *NKX2-5* and *PAX8* promoter mutations, had no manifestation of thyroid dysfunction. Whether this phenotypic variability is due to a variable penetrance or expressivity of the mutant gene or whether the genetic background plays a role remains unclear. Haploinsufficiency, monoallelic expression, or imprinting could all have an impact on the phenotype (Vassart G 2005).

We found no mutations in the *PAX8* or *NKX2-5* genes in our cohort of patients. The only variation we observed was SNP rs199939219 (www.ncbi.nlm.nih.gov/snp) within the *PAX8* gene, that causes for a change of the first nucleotide of the triplet encoding for serine at position 337 (Ser337Ala). However, we do not believe this SNP to be related to TD since the frequency of the SNP within patients and controls was not significantly different.

It is worth to note that the mutations scored in known candidate genes (*NKX2-1, NKX2-5, PAX8* and *TSHR*) in TD patients are certainly an underestimate, considering that mutations have been searched mostly in the coding region and mutations in introns or in regulatory regions may have gone unnoticed, and the same limitations also apply to our study. So far a mutation in one of these candidate genes has been identified in only 3% of patients with thyroid dysgenesis. Moreover a recent report has shown only few non-informative chromosomal aberrations in a cohort of thyroid dysgenesis patients by comparative-genome-hybridization-array (Array-CGH) (Kuhnen, Turan et al. 2014) excluding DNA copy number variations (CNVs) as a frequent cause of thyroid dysgenesis. Epidemiological data collected in a large neonatal screening study of patients with congenital hypothyroidism due to thyroid dysgenesis revealed a striking sporadic occurrence. There was no seasonal or geographic influence, but there was remarkable discordance observed in pairs of monozygotic twins. Furthermore, even in the cases in which mutations in known genes are clearly associated with the disease, a great variability in the phenotype has been observed, even in individuals with the same mutation. Phenotypic variability observed in patients affected by mutations in either *NKX2-1* or *PAX8* genes supports the possibility that other interacting genes may modulate the phenotype. A recent study extends the variable clinical spectrum of patients with *SLC26A4* mutations and points out the necessity to analyze the *SLC26A4* gene in patients with "apparent" thyroid dysgenesis in addition to the known candidate genes *TSHR, PAX8, NKX2.1, NKX2.5* and *FOXE1*. In this work, a homozygous missense mutation (p.Leu597Ser) in the *SLC26A4* gene has been identified in a patient with hypoplastic thyroid tissue, who was otherwise healthy. Based on the unexpected identification of the *SLC26A4* missense mutation in one patient with TD, the *SLC26A4* gene was screened *for* mutations in a cohort of 94 patients with TD who had already been examined for known thyroid dysgenesis candidate genes and CNVs. The authors observed a second case with a homozygous missense mutation (p.Gln413Arg) in the *SLC26A4* gene, who was additionally affected by severe

hearing problems. Both mutations were previously described as loss-of-function mutations in patients with Pendred syndrome and non syndromic EVA (enlarged vestibular aqueduct), but now for the first time have been reported in patients with structural thyroid defects (Kuhnen, Turan et al. 2014). This work confirms the possibility that other interacting genes may modulate the phenotype. A possible multigenic origin for TD was previously suggested in the DHTP mice produced by Amendola and coworkers. To date, several mouse models carrying loss-of-function mutations in thyroid-specific genes have been generated, providing some similarities with the human situation. Recently, a polygenic animal model of congenital hypothyroidism which fulfills all essential criteria of this disease in humans has been described. These mice presented elevated TSH, reduced thyroid hormones, decreased body weight accompanied by thyroid hypoplasia, and increased incidence of thyroid hemiagenesis. The model, characterized by heterozygous null mutations in the genes encoding the transcription factors Nkx2-1 and Pax8, has been named DHTP, for double heterozygous for *Titf1* and *Pax8*. Interestingly, the hypothyroid phenotype is present only when DHTP mice are produced in the B6 strain, whereas it is completely absent in both Sv and DHTP/B6-Sv F1 hybrid mice (Amendola, De Luca et al. 2005). Next, using both association studies and linkage analysis, the authors identified two potential loci linked to the phenotype and finally *Dnajc17*, which encodes for a type III member of the group of Hsp40 (Qiu, Shao et al. 2006) was recognized as a modifier of the thyroid phenotype in DHTP mice (Amendola, Sanges et al. 2010). *Dnajc17* presents a phenylalanine residue at position 273 in the B6 mice, but at the same position in all other mouse strains it shows a tyrosine. Even though the phenylalanine-tyrosine change is a conservative missense mutation, the tyrosine residue at position 273 is conserved in all the *Dnajc17* ortholog genes throughout the evolution from metazoans (Fig. 5)

Dnajc17 is expressed from the early stage of embryonic life in the developing thyroid and remains expressed in the adult thyroid as well as in differentiated thyroid follicular cells in culture. It has been reported that DnaJ proteins interact, via a highly conserved domain (J domain), with Hsp70chaperone proteins and regulate the activity of these chaperones (Qiu, Shao et al. 2006). Chaperone proteins can control the disassembly of transcriptional complexes (Freeman and Yamamoto 2002) and can act as regulators in several gene expression. Since Dnajc17 has a nuclear localization, it could be involved in the regulation of the transcriptional activity of Nkx2-1 and/or Pax8. Furthermore, in a heterologous cellular system, it has been demonstrated not only that Dnajc17 is able to interfere with Tg transcription but also that this activity is sensitive to the replacement of tyrosine by a phenylalanine residue at position 273 (Amendola, Sanges et al. 2010). This result, albeit obtained in an artificial system, suggests that Dnajc17 has the potential to affect transcription of thyroid-specific genes via an interference on Nkx2-1 and thus is in full agreement with its suggested role as modifier of the DHTP phenotype and,

more in general, as one of the genes involved in the multigenic origin of thyroid defects.
All these observations allow to hypothesize a possible role of *DNAJC17* also in the thyroid development. In our work, the entire coding region of the *DNAJC17* gene was screened for mutations in patients with CH due to thyroid dysgenesis in order to evaluate the potential role of this gene in the pathogenesis of TD using HRM technique.
We decided to use HRM because this technique is a simple, rapid and cost effective technique could be used for detection of SNPs in clinical specimens. For constitutional variants, Tindall et al. found that 100% (26/26) of the samples with one or more heterozygous loci were distinguished from wild type by high resolution melting. This compares well to a recent compilation of 19 studies with an overall sensitivity of 99.3% and a specificity of 98.8% (Farrar et al., 2009).
Despite our efforts, we were not able to demonstrate mutations in the *DNAJC17* coding sequence in 45 patients with TD as well as in 100 healthy normal controls. One of the three abnormal melting curve at HRM that we sequenced was determined by the presence of an already known polymorphism c.350A>C. This polymorphism occurs with a frequency of 3% in European normal population as demonstrated by the comparison of the sequence with the NHLBI GO Exome Sequencing Project database.
The absence of *DNAJC17* mutations in our patients well correlate with the complex phenotype observed in *Dnajc17* knock-out mice, that die between the morula and blastocyst stages, data suggesting that this gene could be critical in the early development steps of the mouse embryo. Thus it is possible to hypothesize that alterations in this gene are not compatible with life also in humans as well as in mice. Our results do not exclude the potential role of *DNAJC17* in the pathogenesis of TD, since we screened a low number of patients with TD (only 45) and it necessary to increase the number of patients in order to completely rule out the possible *DNAJC17* role TD.

In conclusion, our search of alterations in already known and novel candidate genes for thyroid dysgenesis resulted in the identification of a novel NKX2-1 mutation in a Brazilian family were the affected members presented the clinical findings of the BTLS. The c.493delC-NKX2-1 mutation has been functionally characterized and we demonstrated that the mutant protein migrates into the nucleus but is not able to transactivate the Tg or SP-C promoters. In addition, a dose-dependent dominant negative effect of the p.Arg165Glyfs*32 was demonstrated only on the Tg promoter, but not on the SP-C promoter. These results indicate that NKX2-1 activity is finely regulated by the interaction of several factors that are still largely unknown. The mechanisms regulating gene expression are different in each tissue.

As a result, no conclusive hypothesis for the pathogenesis of thyroid dysgenesis could be formed. The sporadic occurrence and discordance in

monozygotic twins argue against a classic genetic defect while the stable incidence, even in the presence of opposing environmental conditions, suggests that exogenous pathogenic factors do not play a critical role. It is possible that postmeiotic genetic alterations such as somatic mutations or epigenetic alterations are responsible. Further investigation will hopefully link the different forms of thyroid dysgenesis with specific molecular defects within the regulatory pathways of thyroid development.

| Mutation | | Region | Effect of the mutation | Thyroid Phenothype |
|---|---|---|---|---|
| DNA | Protein | | | |
| c.-456C > T (*) | p.0? | Promoter | Loss of protein binding capacity on the mutant promoter and decreased transcriptional activity | Athyreosis |
| c.287G>A | p.R31H | Paired domain | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia |
| c.286C>T | p.R31C | | Decreased binding to DNA | Severe Hypoplasia |
| c.119A>C | p.Q40P | | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia |
| c.143C>T | p.S48F | | No p300 recruitment and decreased transcriptional activity on Tg promoter | Normal or Athyreosis |
| c.155G>C | p.R52P | | Completely lack of binding to Tg promoter | Hypoplasia |
| c.160A>G(**) | p.S54G | | Decreased binding to TPO promoter and reduced synergism with NKX2-1 on Tg promoter | Hypoplasia |
| c.162C>G | p.S54R | | Decreased DNA binding and transcriptional activity on Tg and TPO promoter | Severe Hypoplasia |
| c.165T>G | p.H55Q | | Decreased transcriptional activity on Tg promoter | Hypoplasia |
| c.170G>A | p.C57Y | | Completely lack of transcriptional activity on TPO promoter | Hypoplasia |
| c.380T>G | p.L62R | | Decreased DNA binding and transcriptional activity on TPO promoter | Hypoplasia |
| c.238_252dup | p.K80-A84dup | | Decreased DNA binding and transcriptional activity on Tg promoter | Hypoplasia |
| c.517C>T | p.R108* | | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia and Ectopia |
| c.398G>A | p.R133Q | | Decreased transcriptional activity on Tg and TPO promoter | Severe Hypoplasia |
| c.674C>T | p. T225M | C-terminus | NO Decreased transcriptional activity on Tg promoter | Ectopia |
| c.1006G>A | p.G336S | | NO Decreased transcriptional activity on Tg promoter | Athyreosis |
| c.989-992delACCC | p.T277fs*1 | | Decreased transcriptional activity on CP5-CAT promoter | Normal or Hypoplasia |

**Table 6**. PAX8 mutations in patients with thyroid dysgenesis (Nettore IC 2013).

| Mutation | | Type of mutation | Mechanism of action of the mutation | Thyroid Phenotype | Other disorders |
|---|---|---|---|---|---|
| DNA | Protein | | | | |
| 2.6-Mb deletion | p.0 | Deletion | Not tested | Absent | Respiratory problems and psychomotor delay |
| Large deletion | | | Haploinsufficiency | Primary hypothyroidism associated with hypoparathyroidism and osteoporosis. | Multisistem disorder of the basal ganglia, salivary glands, bowels and teeth. Respiratory problems and psychomotor delay |
| del 14q11.2q13.3 | | | | CH (Hypoplasia) | Respiratory problems and psychomotor delay |
| del 14q12-13.3 | | | | CH (Normal thyroid) | Respiratory problems, severe developmental delay and psychomotor delay |
| del 14q12-13.3 | | | | Absent | Psychomotor delay |
| del 14q13-21 | | | | CH (Normal thyroid) | Respiratory problems and psychomotor delay |
| c.374-1G >A | p.0? | Splice site mutation | Not tested | CH (Hypoplasia) | Respiratory problems and psychomotor delay |
| c.376-2A>G | | | Haploinsufficiency | CH (Athyreosis) | Respiratory problems |
| | | | | CH (Hemiagenesis) | Respiratory problems |
| | | | | CH (Normal thyroid) | Respiratory problems and psychomotor delay |
| c.255insG | p.G86fs*322 | Frameshift | Haploinsufficiency; Reduced binding and transactivation of Tg | CH (Normal thyroid) | Respiratory problems and psychomotor delay |
| g.1302C>A | p.C87* | Non sense | Haploinsufficiency | CH (Agenesis), | Respiratory problems and psychomotor delay |
| c.609C>A | p.S145* | | | CH (Normal thyroid) | Respiratory problems and psychomotor dela |
| c.470_479delinsGCG | p.P157fs*196 | Frameshift | Haploinsufficiency | CH (Normal thyroid) | Respiratory problems and psychomotor delay |
| c.493C>T | p.R165W | Missense | Dominant positive on SP-C;<br>Reduced transactivation of Tg | CH (Normal thyroid) | Respiratory problems |
| c.493delC | p.R165Gfs*32 | Frameshift | Decreased binding of Tg<br>Reduced transactivation of Tg and SP-C;<br>Dom negative only on Tg but not on SP-C | Subclinical Hypothyroidism | Respiratory problems and psychomotor delay |
| g.2519C>A | p.S169* | Non sense | Haploinsufficiency | CH (Hypoplasia) | Psychomotor delay |
| c.523G>T | p.E175* | | Not tested | CH (Hypoplasia/ Hemiagenesia) | Respiratory problems and psychomotor delay |

| | | | | | |
|---|---|---|---|---|---|
| c.526C>G | p.L176V | Missense | Dom. negative on Tg | CH (Hypoplasia) | Psychomotor delay |
| c.532C>T | p.R178* | Non sense | Decrease binding and reduced transactivation of Tg; not dominant negative on Tg | Absent | Psychomotor delay |
| g.2595_2597insGG | p.A195fs*4 | Frameshift | Haploinsufficiency | CH (Normal thyroid) | Psychomotor delay |
| c.605C>T | p.P202L | Missense | Reduced transactivation of Tg; rescue of activity by co-trasfection with Pax8 | CH (Normal thyroid) | Psychomotor delay |
| g.2626G>T | p.V205F | | Decreased DNA binding on Tg | CH (Hypoplasia) | Respiratory problems and psychomotor delay |
| c.613G>T | p.V205F | | Not tested | CH (not defined) | Psychomotor delay |
| c.619A>T | p.I207F | | Reduced transactivation, dom. negative and decreased DNA binding capacity on Tg and SP-B | CH (Normal thyroid) | Respiratory problems |
| c.621C>G | p.I207M | | Not tested | CH (Normal Thyroid) | Respiratory problems |
| c.629A>C | p.Q210P | | Reduced transactivation of Tg | CH (Hypoplasia) | Psychomotor delay |
| c.650C>A | p.S217* | Non sense | Not tested | CH (not defined) | Respiratory problems, psychomotor delay and psychosis |
| c.713G>T | p.W238L | Missense | Haploinsufficiency | CH (not defined) | Psychomotor delay |
| c.727C>A | p.R243S | | Haploinsufficiency | Absent | Psychomotor delay |
| c.745C>T | p.Q249* | Non sense | Not tested | Absent | Psychomotor delay |
| c.786_787del2 | p.L263fs | Frameshift | Dom. negative on SP-B and SP-C | CH (Normal thyroid) | Respiratory problems and mild psychomotor delay |
| c.825delC | p.P275fs*74 | | Dom. negative on Tg but not on SP-C | CH (Normal thyroid) | Psychomotor delay |
| c.859_860insC | p.Q287fs*121 | | Not tested | CH (not defined) | Respiratory problems and psychomotor delay |
| c.908_909delG | p.G303fs*77 | | Haploinsufficiency | Absent | Psychomotor delay |

**Table 7**. Molecular features of NKX2-1 mutations and clinical characteristics of the patients (Nettore IC 2013).

| Mutation | | Mechanism of action of the mutation | Congenital heart defect | Thyroid phenotype |
|---|---|---|---|---|
| **DNA** | **Protein** | | | |
| c.73C>T | p.R25C | Reduced transactivation on thyroid promoter and dominant negative on WT. | No cardiac malformation | Ectopy/Athyreosis |
| c.355G>T | p.A119S | Reduced transactivation, dominant negative effect and reduced DNA binding on thyroid promoter. | No cardiac malformation | Ectopy |
| | | No functional effects | Congenital hearth defects | Normal |
| c.482G>C | p.R161P | Reduced transactivation, dominant negative effect and reduced DNA binding on thyroid promoter. | Minor mitral valve insufficiency | Ectopy |
| c.795A>C | p.S265R (*) | 30-40% reduced transactivation of the Tg and TPO promoter and dominant negative effect. Normal binding to NKE-2, DIO, Tg and TPO promoter. | No cardiac malformation | Athyreosis |

**Table 8**. NKX2-5 mutations in patients with thyroid dysgenesis. (*) The patient carrying the p.S265R mutation also has a PAX8 promoter (-456C>T) mutation (Nettore IC 2013).

# 6. CONCLUSIONS

Mutations in NKX2-1 have been demonstrated to be responsible for several cases of BTLS. To further identify genetic defects in thyroid dysgenesis we performed a sequencing study of all coding exons of *NKX2-1* in a family from Curitiba, affected by Brain-Lung-Thyroid Syndrome. We also screened 76 patients with persistent CH for known genes (*NKX2-1, NKX2-5, PAX8)* since these are transcription factors involved in thyroid embryogenesis, and new candidate gene, *DNAJC17*. Mutations in the transcription factors *NKX2-1, NKX2-5, PAX8* have been previously described in cases of TD, while there are many observations allow to hypothesize a possible role of *DNAJC17* also in the thyroid development. By SSCP analysis, we found no mutations in the *PAX8* or *NKX2-5* genes in our cohort. The only variation we observed was SNP rs199939219 (www.ncbi.nlm.nih.gov/snp) within the *PAX8* gene, that causes for a change of the first nucleotide of the triplet encoding for serine at position 337 (Ser337Ala). We also performed a functional study for this mutation, but the mutant protein had the same activity of the WT-PAX8, did not produced a dominant negative effect and the activity in presence of NKX2-1, TAZ or P300 is similar to the activity measured for the wild type. This data confirmed that the c.1009T>G *PAX8* was a rare and not pathological polymorphism.

By HRM analysis we screened *DNAJC17* gene, but found only a known polymorphism c.350A>C. Since *Dnajc17* knock-out mice, die between the morula and blastocyst stages, we can hypothesize that alterations in this gene are not compatible with life also in humans as well as in mice.

The sequencing study of all coding exons of *NKX2-1* in a family from Curitiba, affected by Brain-Lung-Thyroid Syndrome, revelead the presence of novel mutation in all members of family. The mutation c.493delC results in a protein of 196 amino acids. The mutant protein contains a frameshift and differs from the WT beginning at position 165, which corresponds to the last amino acid of the NLS. Transfection experiments with the mutant protein demonstrate that the c.493delC protein is synthesized and translocates into the nucleus, but it is unable to bind DNA since it completely lacks the homeodomain. The c.493delC mutant was responsible for a clear dose-dependent dominant negative effect on the WT NKX2-1 when tested on the Tg promoter, whereas when functional assays were performed on the lung SP-C promoter, the c.493delC mutant showed no dominant negative effect.

Our results demonstrate the rare genetic etiology in sporadic cases of TD, at least in genes known to be involved in the formation and migration of follicular cells. Several previously studies in different ethnic populations with TD did not find mutations, either, particularly in sporadic cases (Ramos, Nesi-Franca et al. 2009) (Macchia, Lapi et al. 1998) (Al Taji, Biebermann et al. 2007). It is likely that other epigenetic factors determine TD, such as differential gene

expressions or methylation. This finding make it necessary to carry out further molecular analyses, to better understand the complexities of the genotype–phenotype correlation in the TD.

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# 8. References


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# Selenium in the thyroid: physiology and pathology

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**Summary**

**Selenium (Se) is a trace element that plays a critical role in several processes for human health. The thyroid is the organ with the highest Se content per gram of tissue; in thyroid follicular cells, Se acts as antioxidant by contrasting the production of the reactive oxygen species that are generated during thyroid hormones biosynthesis. In addition, Se is part of the active site of the deoidinases, the enzymes responsible for thyroid hormones activation and inactivation.**
**Herein, the effects of Se supplementation in patients with thyroid related disorders have been reviewed on the basis of the studies published on this issue.**

*KEY WORDS: thyroid, selenium, autoimmunity, antioxidant.*

## Introduction

Selenium (Se) (from the Greek word "Selene" meaning Moon) is an essential trace element, that was discovered in 1817 by the Swedish chemist Jons Jacob Berzelius (1). Two hundred years later, Se was recognized as a fundamental micronutrient that plays a critical role in several processes for human health (2-4): in 1957 Schwarz discovered that traces of Se can prevent liver necrosis in vitamin-E-deficient rats (5) and some years later, in 1973, Rothruck demonstrated the biological function of Se as a cofactor of glutathione peroxidase (GPX) (6).

Se is the most powerful antioxidant agent present in the human body (7), it acts as a growth factor, contributes to the regulation of thyroid hormone biosynthesis, it is a modulator of cardiovascular health, is important in the prevention of neurodegenerative diseases and cancer, and its optimal serum concentrations are required for a correct immune response and fertility (8, 9).
Se plays an important role in human physiology, and it is a basic element for many biological processes. Its most important function is likely to be as a fundamental component of the selenoproteins (4). Selenoproteins are a group of proteins, encoded by at least 25 human genes (10), presenting in their catalytic site in the amino acid selenocysteine (Se-Cys).
The functions of several of the selenoproteins are now known and approximately half of them have been shown to protect the cell against the action of reactive oxygen species (ROS) (10, 11). Selenocysteine-containing proteins influence several biological processes and exhibit a wide range of functions including free radical catabolism, immune response and carcinogenesis (9, 11).
A severe Se deficiency is associated with serious endemic diseases such as Kashin-Beck disease (an osteoarthropathy reported in north-east Asia and Tibet), Keshan disease (a cardiomyopathy encountered in some regions of China), but also mixed ematous cretinism as reported in some areas of central Zaire (12).
Se is present in nature in both inorganic and organic forms (13). Most of the human Se intake originates from diet, but other sources of Se are the drinking water, the environmental pollution, and dietary supplementation. In particular, cereals, organ meats and seafood contains considerable levels of Se in forms of selenocysteine and selenomethionine (14).
Se status varies significantly across different populations and different ethnic groups (4), ranging from severe deficiency to toxic levels (2, 4). The quantity and the type of Se in foods are not constant all over the world (15) and the high variability in Se intake is determined not only by the different Se concentration in the soil (i.e., volcanic grounds have lower Se content), but also by other factors that influence the availability of Se to the food chain or the presence of ions that can complex with Se (2, 4).
The optimal Se intake is still very debate (16) since it has been reported that if a low serum Se can contribute and worsen chronic diseases also Se excesses are associated with toxicity (17-21).
Currently, a definition of the optimal Se intake is based on its role in the modulation of anti-oxidase activity, and the optimal nutritional level are those necessary to maximize the activity of the glutathione peroxidase 1 (GPX1). The optimal intake should bring Se plasmatic

concentration around 95 μg/L. (range 89-114) (2, 16). This corresponds to an intake of 75 and 60 μg/day for men and women, respectively (3, 16). In the US, selenium intake ranges from 60-220 μg/day (21, 22). Se intake in Europe is lower than in the US, with large variability across different countries, ranging from adequate or marginally adequate intakes in Western and Central Europe (30-90 μg/day) to low or deficient intakes in Eastern European countries (7-30 μg/day) (22).

**Role of selenium in thyroid physiology**

The thyroid gland has the highest content of Se per mass unit (23) compared to all other endocrine organs and tissues (4, 10), and the understanding of the fundamental role of Se in the thyroid has been increased significantly during the past few years (10).

Several selenoproteins are expressed in thyroid follicular cells (24-26). Among these, two isoforms of iodothyronine 5'-deiodinase (type1 and type2, DIO1 and DIO2), which produce active thyroid hormone (4); the thioredoxin reductase type 1 (27); the selenoprotein P (27); and three isoforms of glutathione peroxidase, two of which (GPX1 and GPX4) protect thyroid follicular cells from hydrogen peroxide generated by thyroid peroxidase (4), and the third (GPX3) which is present into the lumen, where seems to modulate hydrogen peroxide levels (28).

It is well known that thyroid physiology is closely dependent from the oxidative changes (29): during thyroid hormone biosynthesis, $H_2O_2$ is constantly produced in a considerable amount, thus exposing the thyroid follicular cells to high concentrations of $H_2O_2$ and ROS (1). The peroxidative damage is decreased by the action of the selenoenzymes systems which may be involved in the regulation of hormone biosynthesis (30). Of note, the amount of $H_2O_2$ produced in thyrocytes is similar to the amount that can be produced in activated leukocytes (31), however, while an activated leukocyte's has a life of a few hours, adult human thyrocyte's life spans almost seven years (32). Such a long life requires a very efficient anti-oxidative response process against $H_2O_2$ excess and this protective system is represented by selenoproteins with GPX3 in first line (31, 33, 34). Intra thyroidal Se concentrations are critical for GPXs activity and unbalances in this process as consequence of reduced Se concentrations can increase the oxidative stress and produce damages to the thyroid follicular cells. These damages can determine, to the very end, cell death and hypothyroidism.

**Selenium and autoimmune thyroiditis**

Chronic autoimmune Hashimoto's thyroiditis (HT) is the most common thyroid disorder and it is the main cause of acquired hypothyroidism in iodine-sufficient areas (23). All over the world it has a very high prevalence, affecting about 3% of the population (35). HT is characterized by the presence of auto-antibodies directed to the thyroid epitopes (thyroglobulin and thyroperoxidase), which are closely associated with thyroid dysfunction as consequence of a progressive thyroidal damage and lymphocytic inflammation (36).

HT etiology is still unknown but several aspects are involved in its pathogenesis including genetic predisposition, endogenous and environmental factors, including Se deficiency. A role of Se deficiency was hypothesized by the observation of an higher incidence of HT in areas with severe Se deficiency (37).

Several studies have investigated the possible therapeutic effects of Se administration in patients with HT (12). In 2002, Gartner et al. conducted a randomized, placebo-controlled, blinded trial on 70 female patients in Germany, an area with mild Se and iodine deficiency. The endpoint of the study was to investigate the effects of a short-term Se supplementation on the natural corse of HT. The patients, all under T4 treatment at substitutive doses, were split in two groups: 36 patients received 200 μg of sodium selenite for 3 months, and 34 patients received placebo. In the supplemented patients, serum Se levels increased from 0,87 to 1,09 μM and at the same time anti-TPO antibodies levels dropped by 37% (37).

The same author followed up some of patients for a further 6 months with a further decrease of TPO-Ab levels (38).

In 2003, Duntas et al. reported a 46% drop in thyroid antibodies levels after administration for three months of 200 μg/day of selenomethionine, and a 55,5% drop of antibodies levels after 6 months of treatment (39). Similar results have been reported by Mazokopakis in 2007 with the administration for one year of 200 μg/day of selenomethionine (40).

In 2006, Turker et al. compared the efficacy of 100 μg and 200 μg of selenomethionine supplementation, concluding that a better effect in maximize GPX activities and suppress autoimmune activity is obtained using the higher dose (41).

In contrast with the previous studies, Karanikas et al. found no significant reduction in anti-TPO antibodies after 3 months of Se supplementation with 200 μg/day of sodium selenite in a series of Austrian patients with HT (42).

In 2009, Nacamulli's study focused mainly on the effect of a year-long course of Se supplementation in Italian patients with early-stage HT and a normal thyroid function or mild hypofunction not receiving substitutive L-T4 therapy. 46 patients were treated with 80 μg/day of sodium selenite for 12 months, while 30 patients were given no treatment. TPO-Ab or Tg-Ab level decreased significantly (30% and 19%, respectively) after 12 months in the Se-treated group, but not in the control group (43).

More recently, Krysiak and Okopien conducted a randomized clinical trial involving a group of 170 euthyroid women with recently diagnosed and previously untreated Hashimoto's thyroid and 41 matched healthy subjects. The primary endpoint was to evaluate the effects of L-T4, Selenomethionine, or their combination on several inflammatory markers (TNF-alpha, IL-1B, IL-6, MCP-1, IL-2, INF-G, and high sensitivity CRP). The study demonstrated that L-T4 treatment reduces monocyte release of TNF-A, IL-1B, IL-6, and MCP-1, where-

as selenomethionine inhibits lymphocyte release of IL-2, INF-G, TNF-alpha, and plasma CRP levels. The decrease in cytokines was even strongest when both drugs were administrated together (44).
In 2011, Balazs analyzed IFN-γ-induced HLA-DR expression in cultured human thyrocytes at various concentrations of sodium selenite. Se has a dose-dependent inhibitory effect on the expression of HLA-DR and this effect shows an inverse correlation with anti-oxidative capacity, suggesting that this can be one of the mechanisms associated with the efficacy of Se supplementation in HT (45).
Finally, very recently Anastasilakis et al. described 86 patients with HT and supplemented with 200 μg/day of Se or placebo. No changes in TSH, FT4, FT3 and TPO-Ab levels were detected in Se supplemented patients, while a significant drop in Anti-Tg level (p=0,001) was observed after a 6 months treatment (46).
Results on efficacy of Se supplementation in HT are still not univocal, however it seems that Se may improve the inflammatory activity in patients with HT. Such effect is more evident in areas of mild Se deficiency, but whether this effect is specific for HT or may also be effective in other endocrine autoimmune diseases has yet to be investigated.

### *Selenium and post-partum thyroiditis*

Pregnancy is a period characterized by profound alterations in the biochemical parameters of thyroid gland and thyroid gland, on the other hand, influences the pregnancy. Thyroid autoimmunity is associated with an increased risk of miscarriage, women with elevated TPO-Ab are prone to develop hypothyroxinemia during pregnancy and thyroid dysfunction after delivery (47-49).
Recurrent abortions have been associated with lower serum Se levels (50), and during the 3rd trimester of gestation plasma Se levels drop significantly, returning to baseline after delivery with a risk, for pregnant women of Se deficiency.
Negro et al. reported the effect of Se supplementation on postpartum thyroid status in TPO-Ab positive pregnant women (51). The results indicate that Se supplementation reduced the incidence of post-partum thyroiditis and permanent hypothyroidism, and treated patients showed a significant decrease in the titer of TPO-Ab in the postpartum period, suggesting that selenium administration is effective in prevention of postpartum thyroiditis (PPT). Nevertheless, no other studies so far reported similar results and study replications are required before confirming the efficacy of Se supplementation in prevention of PPT (52).

### *Selenium and Graves' disease*

Graves' Disease (GD), similarly to HT, is organ-specific autoimmune-inflammatory disease with a complex pathogenesis. GD is characterized by lymphocytic infiltration of the thyroid gland with the production of antibodies that bind to the thyrotropin receptor, miming the TSH action (53). The hyperstimulation of the TSH receptor causes an increase of thyroid hormone biosynthesis with $H_2O_2$ over production. This increases ROS production and oxidative stress of the thyroid follicular cell (3). It is now a common view that $H_2O_2$ is implicated in the pathogenesis of GD with an imbalance of the antioxidant/oxidant status, as suggested by several authors (3, 54, 55).
In this view, adequate serum Se levels are fundamental to contrast the inflammatory processes: low serum Se levels have been reported in patients with hyperthyroidism and Graves' disease (56) being associated with the hyperthyroidism, since the administration of antithyroid drugs can increase plasma Se (34, 57). The efficacy of Se supplementation in GD has been suggested by Vrca et al., that demonstrated that patients with GD receiving in addition to methimazole also a supplementation with and antioxidant mixture of Se, beta-carotene, and vitamins C and E, led to euthyroidism faster than patients treated with methimazole alone (58).

### *Selenium and Graves' ophthalmopathy*

Graves' orbitopathy (GO) is caused by inflammation in the orbital connective tissue leading to an enhanced adipogenesis and overproduction of glycosaminoglycans. This causes an increase in orbital volume and fibrosis of the extra ocular muscles (59). Oxidative stress is involved in the pathogenesis of GO (60) as suggested by the results of studies reporting the efficacy of antioxidant treatment in GO.
In 2000, Bouzas et al. demonstrated that 9 of 11 (82%) patients treated with oral antioxidants showed an improvement of mild to moderately severe Graves' ophthalmopathy while improvement was observed only in 3 of 11 (27%) patients in the untreated group ($P < .05$) (61).
A recent study conducted by Marcocci et al. compared the effects on GO of Se administration compared to an anti-inflammatory agent, pentoxifylline, and placebo (62). The results demonstrated that Se treatment was associated with an improved quality of life, less eye involvement and slower progression of GO, compared to placebo and to pentoxifylline. In addition, Se supplementation is associated with less side effects. The study has two limitations: the authors did not measured the effects on plasma Se concentration of Se administration, and the study was conducted in areas of moderate Se deficiency, which may potentiate the effects of Se supplementation.
In addition, no confirmatory studies have been reported, and further trials are necessary to define Se supplementation in the treatment of mild and moderate GO (60).

## Conclusions

In this review the role of the trace element Se was analyzed for its effects on thyroid metabolism and diseases.
Se supplementation seems to produce benefits in the management of autoimmune thyroid disorders (Tab.

**Table 1 - Clinical studies using selenium supplementation in patients with autoimmune thyroid disorders.**

| Disease | Country | Follow-up | Study Gruop | Se dose | Major outcome | Author |
|---|---|---|---|---|---|---|
| HT | Germany | 3 months | 70 patients (F) | 200 μg/day (Se selenite) | Drop in TPO-Ab (-63.3% ) | (37) |
| | Germany | 6 months | 47 patients (F) | 200 μg/day (Se selenite) | Major decrease in TPO-Ab in patients continuing supplementation | (38) |
| | Greece | 6 months | 65 patients (56 F/9 M) | 200 μg/day (SeMe) | Drop in TPO-Ab (-46% at 3 months; -55,5% at 6 months) | (39) |
| | Turkey | 9 months | 88 patients (F) | 100 or 200 μg/day (SeMe) | Drop in TPO-Ab, major effects with 200 μg/day | (41) |
| | Greece | 6 + 6 months | 80 patients (F) | 200 μg/day (SeMe) | Drop in TPO-Ab (-20% at 12 months) | (40) |
| | Austria | 3 months | 36 patients (F) | 200 μg/day (Se selenite) | No effect on TPO-Ab | (42) |
| | Italy | 12 months | 76 patients (65 F/11 M) | 80 μg/day (Se selenite) | Drop in TPO-Ab | (43) |
| | Poland | 6 months | 165 patients | 200 μg/day (SeMe) | Reduction in cytokines production | (44) |
| | Greece | 6 months | 86 patients (53 F/33 M) | 200 μg/day (SeMe) | Drop in TPO-Ab (not significant) | (46) |
| PPT | Italy | Pregnancy and post partum | 232 patients (F) | 200 mcg/day (Seme) | Evaluation of the prevalence of PPTD and hypothyroidism | (51) |
| GD | Croatia | 3 months | 57 patients | Vitamin C and E, Beta carotene and Selenium | Attainment of Euthyroidism | (58) |
| GO | Italy | 6 + 6 months | 159 patients | 200 mcg/day (Selenium) | Improvement GO | (62) |

HT: Hashimoto's thyroiditis; PPT: post-partum thyroiditis; GD: Graves' disease; GO: Graves' ophthalmopathy.

1). Se reduces anti-thyroid antibodies levels and improves thyroid morphology at ultrasound in patients with Hashimoto's thyroiditis, the administration of Se in addition to methimazole seems to be associated with a faster normalization of hyperthyroidism in patients with Graves' disease, and, finally, Se has been proposed as treatment for mild or moderate Graves' orbithopathy.

However, the story is not completely clarified yet and additional studies are required to better define doses and modality of Se supplementation.
In addition, other limitations in understanding the role of Se in thyroid autoimmune diseases are associated with the observation that intrathyroidal Se levels not directly correlate with serum Se concentrations, and, in addition, it should be considered that serum Se assay are expensive and not recommended in routine practice.
Finally, it should be considered that selenium supplementation in subjects with normal selenium levels was associated with an increased risk of type 2 diabetes, and an elevated serum selenium levels were linked to peripheral vascular disease and all-cause mortality in several population studies (20, 21, 63). Although these findings need to be confirmed, long-term selenium supplementation should not be viewed as harmless and a possibly healthy way to prevent illness, at least in patients with normal/high Se income.

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REVIEW ARTICLE

# The molecular causes of thyroid dysgenesis: A systematic review

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**ABSTRACT.** ***Background:*** **Congenital hypothyroidism (CH) is a frequent disease occurring with an incidence of about 1/2500 newborns/year. In 80-85% of the cases CH is caused by alterations in thyroid morphogenesis, generally indicated by the term "thyroid dysgenesis" (TD). TD is generally a sporadic disease, but in about 5% of the cases a genetic origin has been demonstrated. In these cases, mutations in genes playing a role during thyroid morphogenesis (*NKX2-1*, *PAX8*, *FOXE1*, *NKX2-5*, *TSHR*) have been reported.** ***Aim:*** **This work reviews the main steps of thyroid morphogenesis and all the genetic alterations associated with TD and published in the literature.**

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## *INTRODUCTION*

Primary congenital hypothyroidism (CH) is the most frequent endocrine metabolic disease in the infancy, with an incidence of about 1/2500 newborns a year (1). In absence of an adequate treatment, CH is characterized by signs and symptoms of impaired metabolism and by motor and mental handicaps. Before the introduction of a neonatal screening program, CH was one of the most frequent causes of mental retardation. With the exception of the rare cases of central hypothyroidism, CH is characterized by the presence of elevated TSH levels at birth in response to reduced thyroid hormone production.

In the majority of cases (80-85%), CH is due to alterations occurring during the gland organogenesis, which results in a thyroid gland that is absent (thyroid agenesis or athyreosis), hypoplastic (thyroid hypoplasia), or located in an unusual position (thyroid ectopy). All these entities are grouped under the term "thyroid dysgenesis" (TD) (2). In the remaining 15-20% of cases, CH is caused by inborn errors in the molecular steps of thyroid hormones biosynthesis, secretion or recycling. These forms of CH are indicated with the term "dysormonogenesys", and are characterized by an enlargement of the gland (goiter), presumably due to elevated TSH levels. Thyroid dyshormonogenesis shows classical Mendelian recessive inheritance, and the molecular mechanisms in most of these forms have been characterized.

In addition, mostly thanks to the changes in screening strategies for CH of the last years, it has been recently observed an increase number of mild forms of CH associated with eutopic thyroid gland. Of these, about one third of patients presented a permanent hypothyroidism and need to continue $LT_4$ therapy after re-evaluation, and in 8.7% of the studied cases a mutation in TSHR gene has been identified (3).

## *THYROID DEVELOPMENT*

Thyroid morphogenesis starts with the recruitment, in the thyroid anlage, of a group of cells that will form the thyroid follicular cells (TFC). The TFC are the most numerous cells of the thyroid gland that form the thyroid follicles, spherical structures serving as storage of thyroid hormones (4). In mice, thyroid anlage appears at embryonic day (E) 8-8.5 in the ventral wall of the primitive pharynx (5). At E9-E9.5 the endodermal cells of the thyroid anlage start to migrate and to proliferate, invading the surrounding mesenchyme and forming the thyroid bud. Some of the cells during this migration form the thyroglossal duct. At E11.5 the thyroglossal duct disappears, and at E13.5 the thyroid migration is complete.

In humans, the process of thyroid morphogenesis is very similar, lasting from the 3rd to 16th week of gestation (6). The molecular mechanisms controlling the regulation of thyroid cells precursor migration and their proliferation are still largely unknown, but several genes have been suggested to play an important role, including those encoding for the transcription factors Nkx2-1, Foxe1, Nkx2-5, Pax8.

Starting from E8.5 in mouse, the cells fated to become TFC in the primitive pharynx are univocally characterized by the simultaneous expression of *Nkx2-1* (7), *Foxe1* (8), *Pax8* (9), and *Hhex* (10). These factors are also present in other embryonic tissue, but all four are co-expressed only in the thyroid anlage, suggesting that their contemporary expression is required for early stages of thyroid morphogenesis. Therefore, alterations in any of these genes can be responsible for an abnormal thyroid gland morphogenesis and thyroid dysgenesis.

## *THYROID DYSGENESIS*

The most critical events in thyroid organogenesis occur during the first 60 days of gestation in man and the first

*Key-words:* Congenital hypothyroidism, mutation, thyroid dysgenesis, transcription factors, TSH receptor.

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15 days in mice. It is likely that alterations in the molecular events occurring during this period can be associated to TD.
The absence of TFC in orthotopic or ectopic location is called athyreosis. This condition can either be the consequence of lack of formation of the thyroid bud or results from alterations in any of the step following the specification of the thyroid bud and determining a defective survival and/or proliferation of the precursors of the TFC.
The ectopic thyroid is the consequence of a failure in the descent of the developing thyroid from the thyroid anlage to its definitive location in front of the trachea. An ectopic thyroid can be found in any location along the path of the developing gland migration from the *foramen caecum* to the *mediastinum*. In the majority of cases, the ectopic thyroid appears as a mass in the back of the tongue (lingual thyroid, usually functioning). Sublingual ectopic tissues are less frequent; in this case, thyroid tissue is present in a midline position above, below or at the level of the hyoid bone.
Orthotopic and hypoplastic thyroid is reported in 5% of CH cases. Thyroid hypoplasia is probably a genetically heterogeneous form of thyroid dysgenesis. It could be due to defects in any of the genes that control the number of thyroid cells (2).
Thyroid hemiagenesis is a rare congenital abnormality, in which one of the thyroidal lobes fails to develop. Thyroid hemiagenesis is often associated with mild and or transient hypothyroidism but several patients were found to be in euthyroid state. Thyroidal hemiagenesis is predominantly seen in females with an incidence to 0.2% in healthy children. In the large majority of the cases, it is the left lobe to be absent (11). The causes of thyroid hemiagenesis are still unclear and it is unknown whether the disturbance of the lobulation process is due to interference of environmental factors or to genetic abnormality.

## *GENES INVOLVED IN THYROID MORPHOGENESIS*

TD occurs mostly as a sporadic disease; however, a genetic cause of the disease has been demonstrated in about 5% of the reported cases.
TD presents a clear female prevalence (12) and epidemiological studies demonstrated a different incidence of the disease in different ethnic groups (13, 14) suggesting that the genetic background plays a role in this affection. In addition, among the first-degree relatives of patients with sporadic TD, there is a significantly higher rate of asymptomatic thyroid developmental anomalies compared to the normal populations (15). By contrast, against the notion of heritable TD is the finding reported by Perry et al., showing a discordance for TD in 12 of 13 monozygotic twin pairs (16).
Studies on thyroid development in normal and mutated mouse embryos indicate that the simultaneous presence of *Pax8*, *Nkx2-1*, *Foxe1*, and *Hhex* is required for thyroid morphogenesis. Indeed, thyroid dysgenesis is present in animal models with mutations in these genes, and mutations in the same genes have been identified in patients with congenital hypothyroidism associated with TD.

### NKX2-1

NKX2-1 (also known as TTF1) is a transcription factor containing a homeodomain. The human gene maps to chromosome 14q13 and it is formed by 3 exons. In mouse embryos Nkx2-1 is expressed in the thyroid, in the pituitary, in the lung and in the ventral forebrain. Its expression in TFC is constant during all stages of thyroid development and in adulthood (7).
In differentiated TFC, NKX2-1 controls thyroglobulin and thyroperoxidase expression (17). In lung cells it is responsible for the production of the surfactant proteins (18) and in the hypothalamus NKX2-1 acts as transcriptional regulator of genes encoding neuromodulators and hypophysiotrophic peptides, controlling circadian oscillations in GnRH gene transcription (19).
Nkx2-1 null mice die at birth. They are characterized by impaired lung morphogenesis, lack of thyroid and pituitary, and severe alterations in the ventral region of the forebrain (20). Other studies, performed at earlier stage of development demonstated that in *Nkx2-1*$^{-/-}$ embryos the thyroid *primordium* forms in its correct position but subsequently undergoes degeneration and eventually disappears, probably because *Nkx2-1* is required to prevent the initiation of apoptotic process (21). Hence, *Nkx2-1* is essential for the survival of thyroid cells precursor, but it is not necessary for their initial formation.
*NKX2-1* mutations have been described in several patients with primary CH, respiratory distress and benign hereditary chorea, which are manifestations of the "Brain-Thyroid-Lung Syndrome" (BLTS, OMIM # 610978) (Table 1). In the majority of cases haploinsufficiency has been considered to be responsible for the phenotype (22). Only few mutations produce a dominant negative effect on the wild type *NKX2-1* (23-25) and among those in two cases it has been reported a promoter-specific dominant negative effect (24, 25).
All the published *NKX2-1* mutations have variable functional effects, even if the mutations occur in similar regions of the protein. The clinical features of the patients carrying *NKX2-1* mutations are reported in Table 1. Phenotypes are very variable and there is no correlation between the clinical manifestations and the molecular alterations (22).

### Pax8

PAX8 is a transcription factor belonging to the mammalian family of PAX proteins, characterized by the presence of a paired domain (Prd). The human gene encoding for *PAX8* maps to chromosome 2q12-q14 and it consists in ten exons (9, 26).
Homozygous *Pax8*$^{-/-}$ mice are alive at birth but show growth retardation and die within 2-3 weeks if not treated with thyroid hormones (27). The thyroid gland in these mice is severely altered and no follicles and TFC can be detected, while calcitonin-producing C cells are present. In *Pax8*$^{-/-}$ mice at E11.5, thyroid *primordium* appears smaller (hypoplastic thyroid) than in wild-type mice, and at E12.5 follicular cells are essentially undetectable, indicating that Pax8, similarly to Nkx2-1, is required for the survival of thyroid cell precursors but not for their specification (28).

Table 1 - NKX2-1 *mutations in patients with thyroid dysgenesis.*

| Mutation | | Type of mutation | Mechanism of action of the mutation | Thyroid Phenotype | Other disorders | Ref. |
|---|---|---|---|---|---|---|
| DNA | Protein | | | | | |
| 2.6-Mb deletion | p.0 | Deletion | Not tested | Absent | Respiratory problems and psychomotor delay | (48) |
| Large deletion | | | Haploinsufficiency | Primary hypothyroidism associated with hypoparathyroidism and osteoporosis. | Multisistem disorder of the basal ganglia, salivary glands, bowels and teeth. Respiratory problems and psychomotor delay | (49) |
| del 14q11.2q13.3 | | | | CH (Hypoplasia) | Respiratory problems and psychomotor delay | (50) |
| del 14q12-13.3 | | | | CH (Normal thyroid) | Respiratory problems, severe developmental delay and psychomotor delay | (51) |
| del 14q12-13.3 | | | | Absent | Psychomotor delay | (52)(53) |
| del 14q13-21 | | | | CH (Normal thyroid) | Respiratory problems and psychomotor delay | (54)(55) |
| c.374-1G >A | p.0? | Splice site mutation | Not tested | CH (Hypoplasia) | Respiratory problems and psychomotor delay | (56) |
| c.376-2A>G | | | Haploinsufficiency | CH (Athyreosis) | Respiratory problems | (55) |
| | | | | CH (Hemiagenesis) | Respiratory problems | |
| | | | | CH (Normal thyroid) | Respiratory problems and psychomotor delay | (57) |
| c.255insG | p.G86fs*322 | Frameshift | Haploinsufficiency; Reduced binding and transactivation of Tg | CH (Normal thyroid) | Respiratory problems and psychomotor delay | (58) |
| g.1302C>A | p.C87* | Non sense | Haploinsufficiency | CH (Agenesis) | Respiratory problems and psychomotor delay | (50) |
| c.609C>A | p.S145* | | | CH (Normal thyroid) | Respiratory problems and psychomotor delay | (22) |
| c.470_479delinsGCG | p.P157fs*196 | Frameshift | Haploinsufficiency | CH (Normal thyroid) | Respiratory problems and psychomotor delay | (59) |
| c.493C>T | p.R165W | Missense | Dominant positive on SP-C; Reduced transactivation of Tg | CH (Normal thyroid) | Respiratory problems | (23) |
| c.493delC | p.R165Gfs*32 | Frameshift | Decreased binding of Tg Reduced transactivation of Tg and SP-C; Dom negative only on Tg but not on SP-C | Subclinical hypothyroidism | Respiratory problems and psychomotor delay | (25) |
| g.2519C>A | p.S169* | Non sense | Haploinsufficiency | CH (Hypoplasia) | Psychomotor delay | (50) |
| c.523G>T | p.E175* | | Not tested | CH (Hypoplasia/ Hemiagenesia) | Respiratory problems and psychomotor delay | (60) |
| c.526C>G | p.L176V | Missense | Dom. negative on Tg | CH (Hypoplasia) | Psychomotor delay | (55) |
| c.532C>T | p.R178* | Non sense | Decrease binding and reduced transactivation of Tg; not dominant negative on Tg | Absent | Psychomotor delay | (61) |
| g.2595_2597insGG | p.A195fs*4 | Frameshift | Haploinsufficiency | CH (Normal thyroid) | Psychomotor delay | (50) |
| c.605C>T | p.P202L | Missense | Reduced transactivation of Tg; rescue of activity by co-trasfection with Pax8 | CH (Normal thyroid) | Psychomotor delay | (55) |
| g.2626G>T | p.V205F | | Decreased DNA binding on Tg | CH (Hypoplasia) | Respiratory problems and psychomotor delay | (50) |
| c.613G>T | p.V205F | | Not tested | CH (not defined) | Psychomotor delay | (48) |
| c.619A>T | p.I207F | | Reduced transactivation, dom. negative and decreased DNA binding capacity on Tg and SP-B | CH (Normal thyroid) | Respiratory problems | (62) |
| c.621C>G | p.I207M | | Not tested | CH (Normal Thyroid) | Respiratory problems | (63) |
| c.629A>C | p.Q210P | | Reduced transactivation of Tg | CH (Hypoplasia) | Psychomotor delay | (55) |
| c.650C>A | p.S217* | Non sense | Not tested | CH (not defined) | Respiratory problems, psychomotor delay and psychosis | (64) |
| c.713G>T | p.W238L | Missense | Haploinsufficiency | CH (not defined) | Psychomotor delay | (52) |
| c.727C>A | p.R243S | | Haploinsufficiency | Absent | Psychomotor delay | (52) |
| c.745C>T | p.Q249* | Non sense | Not tested | Absent | Psychomotor delay | (65) |
| c.786_787del2 | p.L263fs | Frameshift | Dom. negative on SP-B and SP-C | CH (Normal thyroid) | Respiratory problems and mild psychomotor delay | (23) |
| c.825delC | p.P275fs*74 | | Dom. negative on Tg but not on SP-C | CH (Normal thyroid) | Psychomotor delay | (24) |
| c.859_860insC | p.Q287fs*121 | | Not tested | CH (not defined) | Respiratory problems and psychomotor delay | (66) |
| c.908_909delG | p.G303fs*77 | | Haploinsufficiency | Absent | Psychomotor delay | (52) |

CH: congenital hypothyroidism; Tg: thyroglobulin.

Table 2 - PAX8 *mutations in patients with thyroid dysgenesis.*

| Mutation | | Region | Effect of the mutation | Thyroid Phenothype | Ref. |
|---|---|---|---|---|---|
| DNA | Protein | | | | |
| c.-456C > T (*) | p.0? | Promoter | Loss of protein binding capacity on the mutant promoter and decreased transcriptional activity | Athyreosis | (43) |
| c.287G>A | p.R31H | Paired domain | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia | (67, 68) |
| c.286C>T | p.R31C | | Decreased binding to DNA | Severe hypoplasia | (69) |
| c.119A>C | p.Q40P | | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia | (70) |
| c.143C>T | p.S48F | | No p300 recruitment and decreased transcriptional activity on Tg promoter | Normal or Athyreosis | (71) |
| c.155G>C | p.R52P | | Completely lack of binding to Tg promoter | Hypoplasia | (72) |
| c.160A>G(**) | p.S54G | | Decreased binding to TPO promoter and reduced synergism with NKX2-1 on Tg promoter | Hypoplasia | (73) |
| c.162C>G | p.S54R | | Decreased DNA binding and transcriptional activity on Tg and TPO promoter | Severe hypoplasia | (29) |
| c.165T>G | p.H55Q | | Decreased transcriptional activity on Tg promoter | Hypoplasia | (74) |
| c.170G>A | p.C57Y | | Completely lack of transcriptional activity on TPO promoter | Hypoplasia | (75) |
| c.380T>G | p.L62R | | Decreased DNA binding and transcriptional activity on TPO promoter | Hypoplasia | (67) |
| c.238_252dup | p.K80-A84dup | | Decreased DNA binding and transcriptional activity on Tg promoter | Hypoplasia | (76) |
| c.517C>T | p.R108* | | Completely lack of DNA binding and transcriptional activity on TPO promoter | Hypoplasia and Ectopia | (67) |
| c.398G>A | p.R133Q | | Decreased transcriptional activity on Tg and TPO promoter | Severe hypoplasia | (29) |
| c.674C>T | p. T225M | C-terminus | NO Decreased transcriptional activity on Tg promoter | Ectopia | (77) |
| c.1006G>A | p.G336S | | NO Decreased transcriptional activity on Tg promoter | Athyreosis | (78) |
| c.989-992delACCC | p.T277fs*1 | | Decreased transcriptional activity on CP5-CAT promoter | Normal or Hypoplasia | (79) |

*The patient carrying the c.-456C > T mutation also has a NKX2-5 (p.S265R) mutation. **The patient also displayed unilateral kidney agenesis. CH: congenital hypothyroidism; Tg: thyroglobulin; TPO: thyroperoxidase.



The involvement of *PAX8* has been described in sporadic and familial cases of CH with TD (Table 2). All affected individuals are heterozygous for the mutations and autosomal dominant transmission with incomplete penetrance and variable expressivity has been described for the familial cases. In the majority of cases CH is due to thyroid hypoplasia; however, three patients with athyreosis and two with thyroid ectopy have been described (Table 2). This indicates that in humans, contrary to mice models, both *PAX8* alleles are necessary for correct thyroid morphogenesis, and a reduced dosage of the gene product (haploinsufficiency) causes dysgenesis.

The molecular mechanisms by which PAX8 mutations lead to TD are still unclear. A recent work describes and functionally characterizes two novel PAX8 mutations leading to TD by two different mechanisms. Both mutations (S54R and R133Q) determined a significant reduction in the transcription induction of thyroglobulin and thy-

Table 3 - FOXE1 *mutations.*

| Mutation | | Mechanism of action of the mutation | Human phenotype | Ref. |
|---|---|---|---|---|
| DNA | Protein | | | |
| c.169G>A | p.S57N | Partial lack of DNA binding and transcriptional activity on the Tg promoter | CH, athyreosis, cleft palate | (80) |
| c.194C>T | p.A65V | Complete lack of DNA binding and transcriptional activity on the Tg promoter | CH, athyreosis cleft palate, choanal atresia, bifid epiglottis | (38, 81) |
| c.289T>C | p.W97R | Loss of function of the gene product | CH, thyroid hypoplasia, cleft palate, spiky hair and optional choanal atresia | (82) |
| c.304C>T | p.R102C | Complete lack of DNA binding and transcriptional activity on the TPO promoter | CH, severe thyroid hypoplasia, cleft palate, choanal atresia. | (83) |
| c.394A>G | p.N132D | Slight reduction in binding capability and 5% loss transcriptional activity on the TPO promoter | CH, athyreosis, tongue-tied since birth, poor attention span | (84) |
| c.410T>C | p.F137S | Complete lack of DNA binding and transcriptional activity on the TPO promoter | CH, severe thyroid hypoplasia, cleft palate, and spiky hair | (82) |

CH: congenital hypothyroidism; Tg: thyroglobulin; TPO: thyroperoxidase.

roperoxidase promoters, but while the S54R mutant presented an impaired DNA binding, the DNA-binding properties of R133Q mutation are not affected and the R133Q has no dominant negative effect on the WT protein *in vitro* (29).
Hemiagenesis of the thyroid is a frequent observation in mice double heterozygous for *Nkx2-1*$^{+/-}$ and *Pax8*$^{+/-}$ (30), but, to date, in humans no genetic alterations have been found in patients with thyroid hemiagenesis.

### TSHR

In late stage of thyroid organogenesis, at E15.5, the thyrotropin receptor (Tshr) starts to be expressed in TFC. The binding of TSH to its receptor results in signaling pathway that regulates many functions of the adult thyroid but also during thyroid organogenesis.
TSHR belongs to the G-protein coupled receptors superfamily. The gene encoding TSHR maps to human chromosome 14q31 and to mouse chromosome 12. It consists in ten exons and codify for a 764 aminoacid protein.
The role of the TSHR in thyroid differentiation was first identified in *Tshr*$^{hyt/hyt}$ mice, affected by primary hypothyroidism with elevated TSH and hypoplastic thyroid, as a consequence of a loss of function mutation in the fourth transmembrane domain of TSHR (pro556Leu), which abolishes the cAMP response to TSH (31).
Several patients with homozygous or compound heterozygous loss-of-function *TSHR* mutations have been reported (Table 4). The disease, known as resistance to TSH (OMIM #275200) is inherited as an autosomal recessive trait, and patients are characterized by elevated serum TSH levels, absence of goiter with a normal or hypoplastic gland, and normal to very low serum levels of thyroid hormones. The clinical manifestations are very variable spanning from euthyroid hyperthyrotropinemia to severe hypothyroidism (32).

### FOXE1

FoxE1, also called TTF-2, is a transcription factor containing a forkhead domain and a polialanine stretch of variable length (33-35). The human gene maps to chromosome 9q22 and encodes for a 42kDa protein (33).
In mouse, at an early stage of development, *FoxE1* is present in the posterior *stomatodeum*, in the buccopharyngeal membrane and in Rathke's pouch. At later stages, it appears in the secondary palate, in the definitive choane and in the whiskers and hair follicles (36). In the adult, *FoxE1* is still present in the thyroid, whereas the expression in the esophagus is faint.
Homozygous *FoxE1* knock-out mice are born at the expected ratio but die within 48 h. These mice have no thyroid in its normal location and absence of thyroid hormones. Furthermore, the mice show a severe cleft palate, probably responsible for the perinatal death, and elevated TSH levels in the bloodstream (37).
Studies at the early stages of thyroid morphogenesis demonstrate that at E9.5 in *FoxE1* null embryos, thyroid precursor cells are still on the floor of the pharynx, whereas in wild-type embryos they are detached from the pharynx cavity and begin to descend. At later stages of development, mutant mice exhibit either a small thyroid remnant still attached to the pharyngeal floor or no thyroid gland. It is worth noting that the non-migrating thyroid cells are able to complete their differentiation process as demonstrated by the presence of thyroglobulin. Hence, *FoxE1* plays an essential role in the control of the migration of TFC precursors.
Homozygous mutations in *FOXE1* gene have been reported in patients affected by Bamfort's syndrome (Table 3). This syndrome is characterized by cleft palate, bilateral choanal atresia, spiky hair and athireosis (38). All the affected members carry homozygous missense mutations in conserved amino acids of FOXE1, and the mutant proteins, when tested *in vitro*, show a reduction in both DNA binding and transcriptional activity.
While in mice the absence of *FoxE1* causes either athyreosis or ectopia (37), in humans *FOXE1* mutations have never been associated to thyroid ectopy.

### NKX2-5

In addition to *Nkx2-1*, other genes of the Nkx2 family are present in the primitive pharynx and the thyroid anlage, such as *Nkx2-3*, *Nkx2-5*, *Nkx2-6*.
The gene encoding NKX2-5 in humans is located on chromosome 5q34 and consists of two exons encoding for a 324 aminoacids protein. In early phases of development, *Nkx2-5* is expressed in the ventral region of the pharynx and in thyroid bud; later Nkx2-5 transcript disappears from the thyroid bud, while its expression persists in the heart region (39).
In *Nkx2-5*$^{-/-}$ embryos the thyroid bud is smaller when compared to the wild type, suggesting that Nkx2-5 is required for thyroid development (40).
In humans, NKX2-5 is essential for normal heart morphogenesis, myogenesis, and function (41), and several loss of function mutations in NKX2-5 have been described in patients with congenital heart diseases (42). In addition, heterozigous mutations in *NKX2-5* have been associated to the human ectopic thyroid (40). Functional studies demonstrated that these mutants exhibited a significant functional impairment, with reduction of transactivation properties and dominant negative effect (Table 5).
Recently, it has been reported a girl with athyreosis presenting heterozygous mutations in both *NKX2-5*, and *PAX8* genes (43).
Haploinsufficiency, monoallelic expression, or imprinting could all have an impact on the phenotype (44) and again patients carrying *NKX2-5* mutations show a phenotypic variability in both heart and thyroid phenotype.

### HHEX

Hhex is a homeodomain-containing transcription factor, first identified in multipotent hematopoietic cells. It is encoded by a gene located on chromosome 19 in mice and chromosome 10q23.32 in humans. In embryos, at early stages of development, Hhex is detected in the primitive and definitive endoderm. It is then expressed in the *primordium* of several organs derived from the foregut, including the thyroid bud (10).
Studies of *Hhex*$^{-/-}$ embryos showed that this gene plays a critical role in the development of the liver, forebrain, heart and thyroid (45). In *Hhex* null embryos at E9, the thyroid anlage is present and the expression of *Nkx2-1*,

Table 4 - TSHR *mutations.*

| Position | Protein domain | Mutation | Molecular effects of the mutation | Genotype | Thyroid phenotype | Ref. |
|---|---|---|---|---|---|---|
| Exon 1 | Extracellular domain | p.Q8fsX62 | Extremely low expression at the cell surface. Reduced cAMP production | HET | Normal | (85) |
| | | p.Q33PfsX46 | Nonfunctional protein | HET | Not described | (86) |
| | | p.C41fsX61 | Premature stop with no in vitro expression | HET | Not described | (87, 88) |
| | | p.P27T | Extremely low expression at the cell surface. Reduced cAMP production | HET | Not described | (87) |
| | | p.C31X | Truncated protein | HET | Hypoplasia | (89) |
| | | p.E34K | Reduced cAMP production | HET | Not described | (87, 90) |
| | | p.C41S | Intracellular tapping and decreased binding to TSH | C-HET(p.F525L) | Normal | (91) |
| | | p.R46P | No cAMP production | HET | Not described | (87) |
| Exon 2 | Extracellular domain, leucine-rich region | Exon2del<br>p.P68S | Nonfunctional protein<br>Decreased TSH binding | HOMO<br>C-HET(p.V653L) | Severe hypoplasia<br>Normal | (92)<br>(93) |
| Exon 3 | | p.Q90P^ | Extremely low expression at the cell surface Reduced cAMP production | HOMO<br>C-HET (p.P264S) | Normal | (94) |
| Intron 3 | | g.IVS3+1G>A | Splicing variant, non-functional protein | HOMO | Aplasia | (92) |
| Exon 4 | | p.R109Q | Reduced TSH binding | C-HET (p.W546X) | Normal | (95) |
| Intron 4 | | g.IVS4+1G>A | Splicing variant, non-functional protein | HOM | Thyroid Aplasia | (92) |
| | | g.IVS4+2A>G | Splicing variant, non-functional protein | HET | Not described | (86) |
| Exon 5 | | p.G132R | No TSH binding | C-HET (p.R450H) | Hypoplasia | (96) |
| | | | Partial activity | HET | Normal with decreased uptake of 99mTc | (97) |
| | | p.T145I | Reduced coupling to Gs and Gq | C-HET (p.R450H) | Normal | (98) |
| | | p.I152FsX157 | Truncated protein | HET | Normal | (87) |
| Intron 5 | | g.IVS5-1G>A | Splicing variant, non-functional protein | HOMO | Severe Hypoplasia | (99) |
| Exon 6 | | p.P162A | Reduced biological activity<br>Mild destabilization of the molecule and reduction of TSH binding | C-HET (p.I167N)<br>HOMO<br>C-HET (p.C600R) | Normal<br>Normal<br>Hypoplasia | (91, 100)<br>(92)<br>(101) |
| | | p.P162L | Defected Gq /11 signaling | HET<br>HOMO | Not described<br>Normal | (86)<br>(102) |
| | | p.I167N | No biological activity | C-HET (p.P162A) | Normal thyroid | (100) |
| Exon 7 | | p.A204V | Decreased TSH binding | HET | Normal thyroid | (96) |
| Exon 9 | | p.G245S | Partial activities | HET | Normal with decreased uptake of 99mTc | (103)<br>(97) |
| | | p.L252P | Low expression at the cell surface. Reduced TSH binding and cAMP production | HET | Normal | (87, 104) |
| | | p.P264S | Low expression at the cell surface. Reduced cAMP production | C-HET (p.Q90P) Associated with TPO mutation | Normal | (94) |
| Exon 10 | Extracellular domain | p.R310C | Reduced TSH binding | HOMO<br>HET | Normal<br>Normal | (105)<br>(89) |
| | | p.Q324X | Truncated protein | C-HET (p.D410N) | Slightly enlarged | (91) |
| | | p.C390W | Reduced TSH binding | C-HET(p.W546X) | Slightly enlarged | (91) |
| | | | | HET | Normal | (89) |
| | | p.D403N | Low cAMP production | HET | Hypoplasia | (87, 90, 106) |
| | | | | C-HET (p.R450H) | Not described | (87, 89, 90) |
| | | p.N406fsX424 | Truncated protein, no cell surface expression | C-HET (p.C390W) | Hypoplasia | (107) |
| | | p.D410N | No cAMP production | C-HET (p.G324X) | Slightly enlarged | (91) |
| | | | | HET | Normal | (85) |
| | Intracellular loop #1 | p.Y444X | No cell surface expression, non-functional protein | HOMO | Hypoplasia | (108) |

Table 4 - *Continued.*

| Position | Protein domain | Mutation | Molecular effects of the mutation | Genotype | Thyroid phenotype | Ref. |
|---|---|---|---|---|---|---|
| | | p.R450H | Slight reduction in TSH binding | C-HET (p.G498H)<br>HOMO<br>HET<br>C-HET (p. D403N)<br>C-HET (p. T145I)<br>C-HET (p. I661fsX)<br>C-HET (p.V473I)<br>C-HET (p.R519C)<br>C-HET (p.R519G)<br>C-HET (p.G132R) | Hypoplasia<br>Normal<br>Normal<br>Not described<br>Not described<br>Not described<br>Not described<br>Not described<br>Not described<br>Hypoplastic | (109)<br>(110)<br>(111)<br>(106)<br>(98)<br><br>(111)<br><br>(112) |
| | Transmembrane region #2 | p.Y466C | Reduce cell surface expression. Reduce Gs and Gq/11 signaling. | HET | Not described | (86) |
| | | p.L467P | No cAMP production | HET | Normal | (101) |
| | | p.V473I | Normal TSH binding, Decrease cAMP and IP response | C-HET (p.R450H) | Not described | (112) |
| | Extracellular loop #1 | p.T477I | Low expression at the cell surface. Reduced cAMP production | HOMO | Severe Hypoplasia | (113) |
| | | p.W488R | No cAMP production | HET | Not described | (87, 88, 90) |
| | | p.Q489H | Misfolding of the receptor, immature protein. | HOMO | Athyreosis | (114) |
| | Transmembrane region #3 | p.G498S | Low expression at cell surface | C-HET (p.R450H) | Hypoplasia | (109) |
| | Intracellular loop #2 | p.R519G | Reduced cAMP and IP production | C-HET (p.R450H) | Not described | (112) |
| | | p.R519C | Reduced cAMP and IP production | C-HET (p.R450H) | Not described | (112) |
| | | p.W520X | Truncated protein | HET | Not described | (88) |
| | | p.F525L | No cAMP production | C-HET (p.G324X) | Normal | (91) |
| | | p.F525S | Partial residual activity | HET | Normal with decreased uptake of 99mTc | (97) |
| | | p.M527T | Reduced cAMP production | HET | Not described<br>Normal | (87, 88)<br>(89, 90) |
| | | p.R531Q | Not described | HOMO | Hypoplasia | (102) |
| | | p.R531W | Structural change and/ or reduced basal activity | HET | Normal | (92) |
| | Transmembrane region #4 | p.W546X | Non-functional protein | C-HET (p.C390W)<br>C-HET (p.R109Q)<br>HOMO<br>C-HET (p.A553T) | Slightly enlarged<br>Normal size<br>Normal size<br>Athyreosis | (91)<br>(95)<br>(115)<br>(116) |
| | | p.A553T | Low expression at cell surface<br>Not described<br>Not described<br>Unstable protein, no intermolecular contacts | HOMO<br>C-HET (p.R546X)<br>HET<br>HOMO | Severe hypoplasia<br>Athyreosis<br>Normal<br>Severe hypoplasia | (117)<br>(116)<br>(89)<br>(92) |
| | | p.P556R | Unstable protein, no intermolecular contacts | HOMO | Aplasia | (92) |
| | Transmembrane region #5 | p.I583T<br>p.A593V<br>p.C600R<br>p.Y601H | Reduced Gq/11 signaling<br>Minimal reduction of TSH function<br>No cAMP production<br>No basal activity; Reduced IP production | HET<br>HOMO<br>C-HET (p.P162A)<br>HET | Not described<br>Normal<br>Hypoplasia<br>Hypoplasia | (86)<br>(118)<br>(101)<br>(119-121) |
| | Intracellular loop #3 | p.T607I<br>p.R609Q<br>p.R609X | Reduced Gq/11 signaling<br>Reduced Gq/11 signaling<br>Truncated non-functional protein | HET<br>HET<br>HOMO | Not described<br>Not described<br>Athyreosis/ Severe hypoplasia | (86)<br>(86)<br>(122, 123) |
| | Extracellular loop #3 | p.L653V | No IP but normal cAMP production | HOMO<br>C-HET (p.P68S) | Normal<br>Not described | (124)<br>(93) |
| | | p.T655fsX657 | Truncated protein | C-HET (g.IVS6+3G>C)<br>HET | Athyreosis/ severe hypoplasia<br>Normal | (125)<br>(101) |
| | Transmembrane region #7 | p.I661fsX | Reduction in coupling to Gs and Gq | C-HET (p.R450H) | Normal | (98) |
| | Intracellular tail | p.V689G | Not described | HET | Not described | (103) |

HOMO: homozygous; HET: heterozygous, C-HET: compound heterozygous.

Table 5 - NKX2-5 *mutations in patients with thyroid dysgenesis.*

| Mutation | | Mechanism of action of the mutation | Congenital heart defect | Thyroid phenotype | Ref. |
|---|---|---|---|---|---|
| DNA | Protein | | | | |
| c.73C>T | p.R25C | Reduced transactivation on thyroid promoter and dominant negative on WT | No cardiac malformation | Ectopy/Athyreosis | (40) |
| c.355G>T | p.A119S | Reduced transactivation, dominant negative effect and reduced DNA binding on thyroid promoter | No cardiac malformation | Ectopy | (40) |
| | | No functional effects | Congenital hearth defects | Normal | (126) |
| c.482G>C | p.R161P | Reduced transactivation, dominant negative effect and reduced DNA binding on thyroid promoter | Minor mitral valve insufficiency | Ectopy | (40) |
| c.795A>C | p.S265R (*) | 30-40% reduced transactivation of the Tg and TPO promoter and dominant negative effect Normal binding to NKE-2, DIO, Tg and TPO promoter | No cardiac malformation | Athyreosis | (43) |

*The patient carrying the p.S265R mutation also has a PAX8 promoter (-456C>T) mutation. WT: wild type; Tg: thyroglobulin; TPO: thyroperoxidase.

*Pax8*, and *Foxe1* is not affected. At E10, in the absence of *Hhex*, thyroid budding is severely impaired and the thyroid primordium is represented only by a few non-migrating cells which do not express *Ttf1*, *Pax8*, or *Foxe1* mRNA. At later stages, the primordium disappears. These data strongly suggest that Hhex has no role in thyroid specification but is involved in the survival of already determined thyroid precursors. Since Hhex is required to maintain *Nkx2-1*, *Pax8*, and *Foxe1* expression in the developing thyroid, cannot be excluded that the absence of these factors is the direct cause of the thyroid phenotype displayed by *Hhex*$^{-/-}$ embryos.
To date, no *HHEX* mutations in humans have been described.

## CONCLUSIONS

This review shows that, in some cases, abnormalities in thyroid morphogenesis are due to alterations in genes that regulate the thyroid development. The molecular etiology of the majority of cases of TD still remains largely unclear, and mutations have been identified in only a few cases (46), with a high discrepancy between genotype and phenotype. This discrepancy can be the consequence of different mechanisms of action of the mutation in the different tissues, by the influence of modulating genes (30), but also of the complex interactions between other genetic and/or environmental factors that may modulate the phenotype, as also suggested in animal models (47).
The identification and functional characterization of novel mutations in thyroid transcription factor, known and/or still unknown, may help to understand the transmission mode of disease and provide crucial evidence for genetic counseling.

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# Identification and Functional Characterization of a Novel Mutation in the *NKX2-1* Gene: Comparison with the Data in the Literature

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***Background:*** *NKX2-1* mutations have been described in several patients with primary congenital hypothyroidism, respiratory distress, and benign hereditary chorea, which are classical manifestations of the brain–thyroid–lung syndrome (BTLS).
***Methods:*** The *NKX2-1* gene was sequenced in the members of a Brazilian family with clinical features of BTLS, and a novel monoallelic mutation was identified in the affected patients. We introduced the mutation in an expression vector for the functional characterization by transfection experiments using both thyroidal and lung-specific promoters.
***Results:*** The mutation is a deletion of a cytosine at position 834 (ref. sequence NM_003317) (c.493delC) that causes a frameshift with formation of an abnormal protein from amino acid 165 and a premature stop at position 196. The last amino acid of the nuclear localization signal, the whole homeodomain, and the carboxy-terminus of NKX2-1 are all missing in the mutant protein, which has a premature stop codon at position 196 (p.Arg165Glyfs*32). The p.Arg165Glyfs*32 mutant does not bind DNA, and it is unable to transactivate the thyroglobulin (Tg) and the surfactant protein-C (SP-C) promoters. Interestingly, a dose-dependent dominant negative effect of the p.Arg165Glyfs*32 was demonstrated only on the Tg promoter, but not on the SP-C promoter. This effect was also noticed when the mutation was tested in presence of PAX8 or cofactors that synergize with NKX2-1 (P300 and TAZ). The functional effect was also compared with the data present in the literature and demonstrated that, so far, it is very difficult to establish a specific correlation among *NKX2-1* mutations, their functional consequence, and the clinical phenotype of affected patients, thus suggesting that the detailed mechanisms of transcriptional regulation still remain unclear.
***Conclusions:*** We describe a novel *NKX2-1* mutation and demonstrate that haploinsufficiency may not be the only explanation for BTLS. Our results indicate that NKX2-1 activity is also finely regulated in a tissue-specific manner, and additional studies are required to better understand the complexities of genotype–phenotype correlations in the NKX2-1 deficiency syndrome.

## Introduction

NK2 homeobox 1 (NKX2-1, also called TTF-1; MIM# 600635) was initially identified in rat thyroid cells as a 42 kDa phosphorylated nuclear protein able to bind to the thyroglobulin (Tg) promoter (1). It belongs to the NKX2 class of transcription factors and is encoded by a three-exon structured gene located on chromosome 14q13. During human development, the gene is expressed in the ventral diencephalon, telencephalon, lung bud, and the thyroid primordium (2,3). *Nkx2-1* null mice lack thyroid and pituitary glands, have lung hypoplasia with severe respiratory failure, and have defects in the ventral forebrain (4).

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The association of choreoathetosis, hypothyroidism, and pulmonary alterations was linked to mutations in the gene encoding NKX2-1 in the so-called brain–thyroid–lung syndrome (BTLS).

A novel *NKX2-1* mutation was identified and functionally characterized in a family with five affected patients in two generations showing hypothyroidism, benign hereditary chorea, and respiratory distress. All the affected members presented a heterozygous deletion of the cytosine at position 834 (ref. sequence NM_003317) of the *NKX2-1* gene (c.493delC). The mutation is responsible for a frameshift that produces an abnormal protein from amino acid 165 with a premature stop codon at position 196.

## Materials and Methods

### *Patients*

The family included five affected patients in two generations living in Curitiba, Brazil (Fig. 1). All family members gave their informed consent for the study. The study was approved by the ethical committee of each participating institution. The clinical characteristics of the affected members of the family are reported in the Results section.

### *DNA amplification and sequencing*

Genomic DNA was extracted from peripheral blood leukocytes of all family members. The three exons of the *NKX2-1* gene were amplified by polymerase chain reaction (PCR) as previously described (5). PCR products were purified with Antarctic phosphatase-exonuclease I (New England BioLabs, Ipswich, MA) at 37°C for 15 minutes and 80°C for 15 minutes for the enzyme activation and inactivation respectively and the products were bidirectionally sequenced with a 377 ABI sequencer (Applied Biosystems, Foster City, CA). The genes encoding PAX8 (6), NKX2.5 (7), and TAZ (8) were also amplified and sequenced as previously described. To exclude the presence of polymorphysms, 100 normal subjects were also screened as controls.

### *Mutagenesis*

The mouse *Nkx2-1* gene from the p3XFLAG-CMV-10-NKX2-1 vector was used as template in a PCR with the sense primer 5′-CCG CCG GAA GCG CGG GTG CTC TTC T-3′ and the antisense primer 5′-GAG AAG AGC ACC CGC GCT TCC GGC-3′ using the QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, La Jolla, CA) according to the manufacturer's procedure. The presence of the mutation in the obtained vector was confirmed by direct sequencing.

### *Western blot*

HEK293 cells were transiently transfected with 10 $\mu$g of either wild-type (WT)-*Nkx2-1* or c.493delC-*Nkx2-1* plasmids using the FuGene 6 Transfection Reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions. After 48 hours, cells were scraped in phosphate-buffered saline (pH 7.4) and centrifuged at 1792 $g$ for 5 minutes at 4°C. Pellets were frozen and stored at −80°C until used to prepare nuclear or total extracts as previously described (5).

Ten micrograms and 40 $\mu$g each of nuclear and total extracts were boiled in Laemmli buffer and resolved on a 12% SDS-PAGE gel. The gel was blotted on Immobilon P (Millipore Corp., Bedford, MA) for 90 minutes at a constant current of 100 mA. Immunodetection of NKX2-1 was performed by using a monoclonal anti-FLAG antibody (M2; Sigma, St. Louis, MO) diluted 1:3000 in Tris-buffered saline containing 5% nonfat milk (Bio-Rad Laboratories, Inc., Richmond, CA), and the filter was treated with a 1:3000 dilution of goat anti-mouse IgG conjugated to horse radish peroxidase (Bio-Rad).

### *Electrophoretic mobility shift assay*

The binding to DNA of the c.493delC mutant was studied by electrophoretic mobility shift assay (EMSA) using double-stranded end-labeled oligonucleotide C for the Tg promoter as described (9), and double-stranded end-labeled oligonucleotide C2 for the surfactant protein C (SP-C) promoter (10).

### *Cell culture and* in vitro *functional assays*

HEK293 and HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 4.5 g/L D-glucose, 50 U/mL penicillin, 50 $\mu$g/mL streptomycin, and 10% fetal bovine serum under humidified 5% carbon dioxide/95% air at 37°C and grown in 24-well plates to 70%–80% confluence (*i.e.*, $\sim 5 \times 10^4$ cells per well).

HeLa cells were transfected with the reporter plasmid (0.1 $\mu$g of pTg-Luc, 0.3 $\mu$g of SP-C-Luc 0.32) plus 3 ng of CMV-Ren as an internal control vector, and different doses of effector plasmids. HEK293 cells were transfected with 0.1 $\mu$g of the reporter pTg-Luc plasmid and 1.5 ng of CMV-Ren internal control vector plus different doses of effector plasmids. In each set of transfection experiments, the total amount of transfected DNA was normalized using an empty vector.

Transfections were performed with the TurboFect *in vitro* Transfection Reagent (Fermentas Life Sciences, Pittsburgh, PA), using a DNA/Turbofect ratio of 1:2 in all experiments. Cells were harvested after 36–48 hours and analyzed sequentially for firefly and Renilla luciferase activities (Dual-Luciferase Reporter Assay System, Promega, Madison, WI).

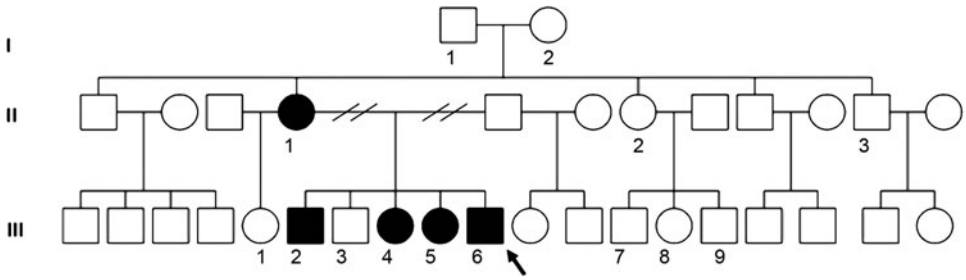


**FIG. 1.** Pedigree of the studied family. The family members carrying the c.493delC mutation (p.Arg165Glyfs*32) and showing the clinical manifestation of brain–thyroid–lung syndrome (BTLS) are in black. The proband (III-6) is indicated by an arrow.

The ratios between the measured firefly and Renilla luciferase activities were expressed relative to the ratios obtained in cells transfected with the reporter and an empty expression vector (CMV-Flag) only. Transfection experiments were performed in duplicate and repeated at least three times. Data are shown as mean ± SD. Statistical analysis was performed using Student's *t*-test.

## Results

### *Patients*

The family included five affected patients in three generations (Fig. 1). Collection of clinical information was difficult because the members of the family live in different towns; therefore, some of the clinical characteristics were missing.

The proband (III-6) was the fifth child of nonconsanguineous parents. He was born at term with a birth weight of 2820 g, but a delay in motor development was soon evident: he took his first steps at 24 months of age. During infancy, the proband showed moderate generalized choreiform movements and cerebellar ataxia that remained stable thereafter, accompanied by several episodes of respiratory insufficiency that were diagnosed either as asthma or pneumonia. Routine blood chemistry and hematology tests and electrocardiograms were normal. Brain MRI, electromyography, and muscle biopsy were also normal.

When the proband was 13 years old, his thyroid function tests (TFTs) revealed subclinical hypothyroidism: serum thyrotropin (TSH) was 9.1 mU/L (normal range 0.35–4.94 mU/L), free thyroxine ($FT_4$) was 1.07 ng/dL (normal range 0.7–1.48 ng/dL) and triiodothyronine ($T_3$) was 127.55 ng/dL (normal range 58–159 ng/dL). Serum antithyroid antibodies tests were negative.

The proband's mother (II-1) presented with short stature, mild generalized choreiform movements since infancy, subclinical hypothyroidism (TSH 8.51 mU/L, $FT_4$ 1.07 ng/dL, $T_3$ 109.46 ng/dL), and episodes of respiratory insufficiency (diagnosed as asthma) in childhood.

Subject III-2, a brother of the proband, presented with cervical dystonia, moderate generalized choreiform movements, and hypothyroidism (TFT not available) at the age of 22 years.

Subject III-4 (the oldest sister of the proband) was diagnosed with a mild choreiform movement disorder at the age of 17 years. At that time, subclinical hypothyroidism (TSH 8.13 mU/L, $FT_4$ 1.1 ng/dL, $T_3$ 122.08 ng/dL) was also noticed. She was treated with 25 $\mu$g/d of L-thyroxine, which normalized serum TSH levels.

The proband's youngest sister (III-5) presented with moderate generalized choreiform movements, mild cognitive deficit, and subclinical hypothyroidism (TSH 5.43 mU/L, $FT_4$ 1.19 ng/dL, $T_3$ 126.60 ng/dL) at the age of 14 years.

All the other members in the family (I-1, I-2, II-2, II-3, III-1, III-3, III-7, III-8, III-9) had normal TFTs and normal thyroid morphology on ultrasound examination.

### *Sequencing*

Sequencing of the *NKX2-1* coding region of the proband's DNA revealed a new monoallelic deletion of the cytosine at position 834 (accession no. NM_003317.3). The mutation is responsible for a frameshift that produces an abnormal protein from amino acid 165. The last amino acid of the nuclear localization signal (NLS), the whole homeodomain, and the carboxy-terminus of NKX2-1 are all missing in the mutant protein, which has a premature stop codon at position 196 (p.Arg165Glyfs*32). The mutant protein has an approximate molecular weight of 20 kDa.

The same mutation (TRANSNP_1331899076), never described before, was present in all the affected members of the family but not in any of the unaffected members of the family or in the 100 normal controls.

No mutations were detected in the coding sequences of *PAX8*, *NKX2-5*, and *TAZ* genes in the family members or in the 100 normal controls.

### *Nuclear localization*

Since the c.493delC mutation occurs in the codon encoding for the last amino acid of the NLS of NKX2-1, we tested the effect on the nuclear localization. To this aim, HeLa cells were transfected with either the WT-*Nkx2-1* or the c.493delC-*Nkx2-1*. After 24 hours, both nuclear and cytoplasmatic proteins were extracted from transfected cells and Western blot results are shown in Figure 2.

As expected, WT-NKX2-1 was synthesized and migrated into the nucleus, disappearing from the cytosolic extracts. Despite the alteration of the nuclear localization signal, the c.493delC was able to migrate into the nucleus.

These results indicate that the p.Arg165Glyfs*32 was synthesized and was capable of migrating into the nucleus.

### *DNA binding capacity*

DNA binding properties of the c.493delC mutant were also analyzed by EMSA using a short DNA stretch corresponding to the NKX2-1 binding site within the Tg promoter (oligo C) (9) and the SP-C promoter (oligo C2) (10). The results are shown in Figure 3. A retarded band could be detected with the WT, while the c.493delC NKX2-1 was, as expected, unable to bind to DNA because of the absence of the homeodomain. Moreover, the addition of the same or double amount of c.493delC extracts did not reduce the binding of WT-NKX2-1. This indicates that c.493delC-NKX2-1 was unable to physically interact and modulate WT-NKX2-1 binding to DNA. The specificity of all the observed complexes was demonstrated in competition experiments adding a 100-fold excess of cold oligos.

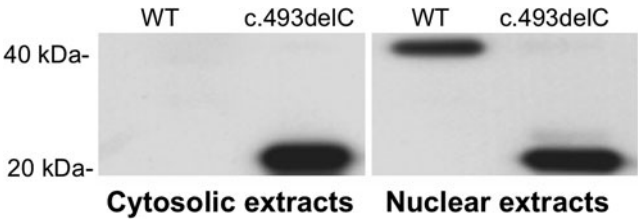


**FIG. 2.** Western blot analysis with the anti-FLAG antibodies of cytosolic and nuclear extract of the cells transfected with both wild-type (WT) and c.493delC (p.Arg165Glyfs*32) cloned into the *p3XFLAG-CMV-10* plasmid. The c.493delC is normally synthesized and able to migrate into the nucleus.

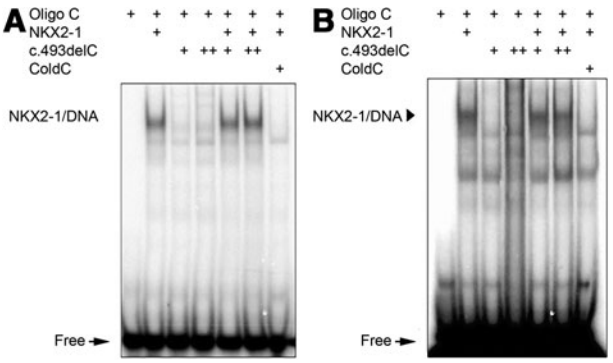


**FIG. 3.** DNA binding capability of the c.493delC mutant (p.Arg165Glyfs*32). Electrophoretic mobility shift assay (EMSA) was performed with nuclear extracts of HEK293 cells transfected with WT and c.493delC constructs on the thyroglobulin (Tg) promoter **(A)** and surfactant protein C (SP-C) promoter **(B)**. Arrowheads indicate the bound NKX2-1 to the oligos. The co-transfection of the WT-*Nkx2-1* with the same (+/+) or double (+/++) amount of the mutant c.493delC does not reduce the binding of the WT. Extracts were also incubated with a large excess (100-fold) of cold oligos as a competitor that produced a significant displacement of the bound labeled probe.

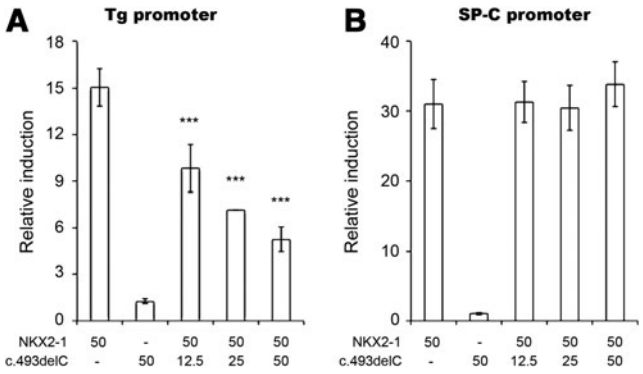


**FIG. 4.** Transcriptional activation of Tg and the SP-C promoters. **(A)** Effects of transfection of WT-NKX2-1 and c.493delC-NKX2-1 on the Tg promoter. Addition of increasing amounts (12.5, 25, 50 ng) of c.493delC to a constant amount (50 ng) of the WT produced a significant decrease in the promoter activity (***$p < 0.001$). **(B)** Effects of transfection of WT-*Nkx2-1* and c.493delC-*Nkx2-1* on the SP-C promoter. Contrary to what was observed for Tg, addition of increasing amounts (12.5, 25, 50 ng) of c.493delC to a constant amount (50 ng) of the WT did not reveal a dominant negative effect.

### *Transactivation capacity of mutated and WT NKX2-1 proteins*

The transcriptional properties of both WT- and c.493delC-NKX2-1 were investigated by co-transfection assays as previously described. As expected, the c.493delC mutant was unable to activate both the Tg (11) and the SP-C promoters (12).

Thereafter co-transfection experiments were performed using both WT and the c.493delC mutant on the same promoters. Interestingly we were able to demonstrate a dose-dependent dominant negative effect of the c.493delC mutant on the WT-NKX2-1 when used with the Tg promoter (Fig. 4A), but no dominant negative effect could be demonstrated on the SP-C promoter (Fig. 4B), indicating that the c.493delC mutant produced a dominant-negative effect on the wild-type NKX2-1 in a promoter-specific manner.

To further understand the dominant-negative action and investigate the c.493delC effects, co-transfection experiments were performed with PAX8, P300, and TAZ on the Tg promoter.

PAX8 (Paired Box Gene 8) is a transcription factor expressed in the thyroid but not in the lung. It synergizes with NKX2-1 to stimulate Tg and thyroperoxidase promoter activities (13).

P300 (E1A-Binding Protein), a general transcriptional coactivator, plays a pivotal role in the activity of many transcription factors, either by bridging sequence-specific DNA-binding factors with elements of the basal transcriptional machinery, such as transcription factor IIB and polymerase 2, or by its intrinsic histone acetyltransferase activity. NKX2-1, PAX8, and P300 physically interact with each other (13–15), and the functional synergism may involve the direct cooperation of all three factors. The addition of P300 increases the transactivation properties of PAX8, NKX2-1, and both factors together.

TAZ (transcriptional co-activator with a PDZ-binding motif, also known as WWTR1) is also present in thyroid tissue and in differentiated thyroid cell lines (16), thus acting as a potent co-activator for both PAX8 (16) and NKX2-1 (17).

As previously shown (13), we were able to demonstrate that PAX8 synergized with the WT protein and increased Tg promoter activity. The co-transfection with the c.493delC did not modify the PAX8 basal activity, and the dominant negative effect was also present when both WT and c.493delC proteins were transfected with PAX8 (Fig. 5).

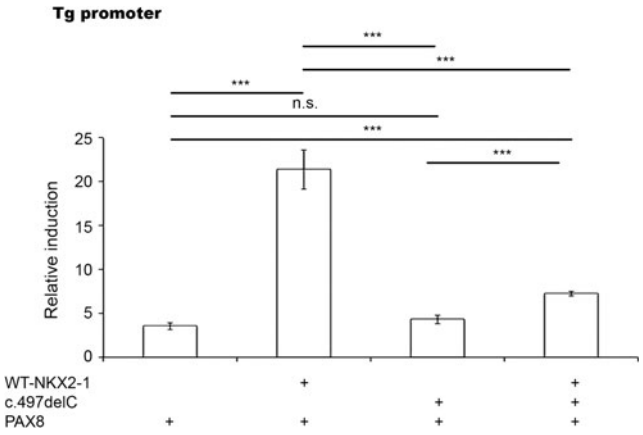


**FIG. 5.** Synergism with PAX8. Transcription activation of the Tg promoter by WT-NKX2-1 and c.493delC-NKX2-1 in the interaction with PAX8. HeLa cells have been transfected with 50 ng of each plasmid and 100 ng of the reporter. The addition of WT produced a significant (***$p < 0.001$) increase in the PAX8 activity, while the addition of the c.493delC had no effect. If both WT-*Nkx2-1* and c.493delC-*Nkx2-1* were transfected, the dominant negative effect of the mutant on the WT protein was still present. The presence of PAX8 did not rescue the dominant negative effect on the Tg promoter.

To further study the interplay of P300, PAX8, and both WT and c.493delC proteins, co-transfection experiments were performed in HEK293 cells, since these cells lack the endogenous P300 (18). Again, if the c.493delC was added to the cells, there was no increase in the activation produced by PAX8 and/or P300, suggesting that this truncated protein did not interfere with the complex. Finally, when all four factors were present (WT, c.493delC, PAX8 and P300), a slight decrease in the activity could be observed compared with the multiprotein complex of NKX2-1/PAX8/P300 (Fig. 6).

These results indicate that the dominant negative effect can also be observed when a multifactorial complex is present, and PAX8 or P300 are unable to rescue the effect of the mutation.

Similar results were obtained when the coactivator TAZ was used in HeLa cells (data not shown).

## Discussion

Mutations in *NKX2-1* have been demonstrated to be responsible for several cases of BTLS, and in this article we describe a novel mutation in the gene encoding for NKX2-1 in a family with the clinical manifestations of benign hereditary chorea, hypothyroidism, and respiratory distress. The mutation c.493delC results in a protein of 196 amino acids. The mutant protein contains a frameshift and differs from the WT beginning at position 165, which corresponds to the last amino acid of the NLS. Transfection experiments with the mutant protein demonstrate that the c.493delC protein is synthesized and translocates into the nucleus, but it is unable to bind DNA since it completely lacks the homeodomain. The c.493delC mutant was responsible for a clear dose-dependent dominant negative effect on the WT NKX2-1 when tested on the Tg promoter, whereas when functional assays were performed on the lung SP-C promoter, the c.493delC mutant showed no dominant negative effect.

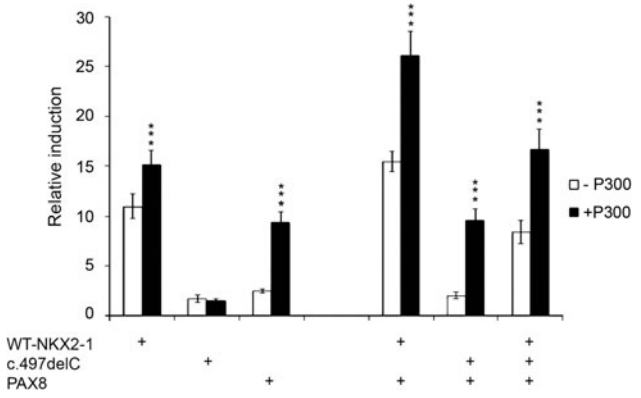


**FIG. 6.** Effect of P300. HEK293 cells were co-transfected with WT-*Nkx2-1* (50 ng), c.493delC-*Nkx2-1* (50 ng), *PAX8* (50 ng), and *P300* (300 ng) expression vectors, and the pTg-Luc reporter (100 ng) in the indicated combinations. The addition of P300 significantly increased the activity of the Tg promoter in response to WT-NKX2-1 and PAX8, but had no effects on the activity of c.493delC. The cooperation between WT, PAX8, and P300 produced an additional increase in the activity, while the activation of the complex c.493delC/PAX8 did not differ from the activation observed when PAX8 was co-transfected with P300 alone. Finally, when all the factors were present, there was a reduction of the activation compared with the WT + PAX8 complex, as a consequence of the dominant negative effect of the c.493delC mutant on the WT. Significant differences between the samples with and without P300 are indicated: ***$p < 0.001$.

Several mutations in *NKX2-1* have been reported (5,19–38), and in the majority of cases haploinsufficiency has been considered to be responsible for the phenotype, as also suggested by the finding of motor abnormalities and mild hyperthyrotropinemia in the *Nkx2-1* heterozygous knockout mice (19). Only a few mutations (Table 1) produce a dominant negative effect on the WT NKX2-1 (20,21), and there is only one case among them describing a promoter-specific dominant negative effect (22). In this last case, contrary to what was observed for the c.493delC, the mutant protein contained the homeodomain and could bind to DNA. The authors suggested that the possible interference of the mutant NKX2-1 (p.P275fs*74) with the cooperative interaction between NKX2-1 and PAX8 or NKX2-1 and other cofactors on the Tg promoter is the cause of the promoter-specific dominant negative effect. Since our mutant did not bind to DNA, it is probable that the amino terminus of NKX2-1 binds to other factors that are relevant for its activity on the Tg promoter, thus excluding them from the transcriptional complex and reducing the activity of the WT NKX2-1. In fact, tissue-specific transcriptional regulation is achieved by the combined interaction among transcription factors, co-regulators, and other components regulating basal transcription. In the thyroid, NKX2-1 physically interacts with both PAX8 (13) and TAZ (16). In addition P300, a ubiquitous transcriptional cofactor, has been demonstrated to play an important role for the NKX2-1/PAX8 synergy (14,39). PAX8, P300, and TAZ were tested to see whether they could influence the transactivation of NKX2-1 on its target promoters. The presence of these cofactors increased the activation of the Tg and SP-C promoters, but did not change the thyroid-specific dominant negative effect. In addition, no rescue of the NKX2-1/PAX8 synergism was observed, contrary to what was previously reported in the case of a PAX8 mutation (39).

It has been suggested that NKX2-1 binds to DNA as a dimer (40) and that the interaction with PAX8 occurs via the amino terminus of the NKX2-1 protein (13). Several other factors, most of which not yet defined, may be involved in the regulation of transcription. We believe that the truncated NKX2-1 mutant described in this article interferes with the activity of WT-NKX2-1, decreasing the activity of the multiprotein transcriptional complex. It is likely that PAX8 plays a critical role in modulating this effect in thyroid cells. In lung cells, where PAX8 is absent, the dominant negative effect can not be observed. Even if HeLa cells do not express PAX8, it has been previously suggested that other proteins with a similar activity may replace the function of PAX8 on the Tg promoter (22).

Very recently Silberschmidt and coworkers (41) produced mice lacking one of the two redundant activation domains of *Nkx2-1*, or having defective phosphorylation of the protein. They demonstrated that each mutant shows a distinct phenotype. They also revealed a level of complexity that could not be predicted by experiments carried out in cultured cells and stressed the discrepancy between the results in the cell models and in those that can be obtained in whole organisms (41). In this context, the major molecular effect of our mutation on the Tg promoter is dominant, while hypothyroidism is only mild (subclinical) in all the affected members of our

Table 1. Molecular Features of *NKX2-1* Mutations and Clinical Characteristics of the Patients

| *Mutation* | | *Mechanism of action of the mutation* | *Type of mutation* | *Hypothyroidism* | *Respiratory problems* | *Psychomotor delay* | *Reference* |
|---|---|---|---|---|---|---|---|
| *DNA* | *Protein* | | | | | | |
| c.374-1G>A | p.0? | Not tested | Splice site mutation | Yes | Yes | Yes | (37) |
| c.613G>T | p.V205F | Not tested | Missense | Yes | No | Yes | (38) |
| c.523G>T | p.E175* | Not tested | Nonsense | Yes | Yes | Yes | (32) |
| c.650C>A | p.S217* | Not tested | Nonsense | Yes | Yes | Yes | (23) |
| c.745C>T | p.Q249* | Not tested | Nonsense | No | No | Yes | (24) |
| c.859_860insC | p.Q287fs*121 | Not tested | Frameshift | Yes | Yes | Yes | (33) |
| 2.6 Mb deletion | p.0 | Not tested | Deletion | No | Yes | Yes | (38) |
| c.376-2A>G | p.0? | Haploinsufficiency | Splice site mutation | Yes | Yes | No | (20) |
| c.376-2A>G | p.0? | Haploinsufficiency | Splice site mutation | Yes | Yes | Yes | (31) |
| c.713G>T | p.W238L | Haploinsufficiency | Missense | Yes | No | Yes | (30) |
| c.727C>A | p.R243S | Haploinsufficiency | Missense | No | No | Yes | (30) |
| g.1302C>A | p.C87* | Haploinsufficiency | Nonsense | Yes | Yes | Yes | (29) |
| c.609C>A | p.S145* | Haploinsufficiency | Nonsense | Yes | Yes | Yes | (5) |
| g.2519C>A | p.S169* | Haploinsufficiency | Nonsense | Yes | No | Yes | (29) |
| c.255insG | p.G86fs*322 | Haploinsufficiency; reduced binding and transactivation of Tg | Frameshift | Yes | Yes | Yes | (19) |
| c.470_479delinsGCG | p.P157fs*196 | Haploinsufficiency | Frameshift | Yes | Yes | No | (35) |
| g.2595_2597insGG | p.A195fs*4 | Haploinsufficiency | Frameshift | Yes | No | Yes | (29) |
| c.908_909delG | p.G303fs*77 | Haploinsufficiency | Frameshift | No | No | Yes | (30) |
| del14q13 | p.0 | Haploinsufficiency | Deletion | Yes | Yes | Yes | (20) |
| del 14; q11.2q13.3 | p.0 | Haploinsufficiency | Deletion | Yes | Yes | Yes | (29) |
| del 14q12–13.3 | p.0 | Haploinsufficiency | Deletion | Yes | Yes | Yes | (28) |
| del 14q13–21 | p.0 | Haploinsufficiency | Deletion | Yes | Yes | Yes | (27) |
| del 14q12–13.3 | p.0 | Haploinsufficiency | Deletion | No | No | Yes | (30) |
| del 14q12–13.3 | p.0 | Haploinsufficiency | Deletion | No | No | Yes | (25) |
| Large deletion | p.0 | Haploinsufficiency | Deletion | Yes | Yes | Yes | (34) |
| c.493C>T | p.R165W | Dominant positive on SP-C; reduced transactivation of Tg | Missense | Yes | Yes | No | (21) |
| c.526C>G | p.L176V | Dominant negative on Tg | Missense | Yes | No | Yes | (20) |
| c.605C>T | p.P202L | Reduced transactivation of Tg; rescue of activity by co-transfection with Pax8 | Missense | Yes | No | Yes | (20) |
| g.2626G>T | p.V205F | Decreased DNA binding on Tg | Missense | Yes | Yes | Yes | (29) |
| c.619A>T | p.I207F | Reduced transactivation, dominant negative and decreased DNA binding capacity on Tg and SP-B | Missense | Yes | Yes | No | (36) |
| c.629A>C | p.Q210P | Reduced transactivation of Tg | Missense | Yes | No | Yes | (20) |
| c.532C>T | p.R178* | Decrease binding and reduced transactivation of Tg; not dominant negative on Tg | Nonsense | No | No | Yes | (26) |
| c.493delC | p.Arg165Glyfs*32 | Decreased binding of Tg; reduced transactivation of Tg and SP-C; dominant negative only on Tg but not on SP-C | Frameshift | Yes | Yes | Yes | Present work |
| c.786_787del2 | p.L263fs | Dominant negative on SP-B and SP-C | Frameshift | Yes | Yes | Mild | (21) |
| c.825delC | p.P275fs*74 | Dominant negative on Tg but not on SP-C | Frameshift | Yes | No | Yes | (22) |

family. This discrepancy can be the consequence of different mechanisms of action of the mutation in the different tissues, but also of the complex interactions between other genetic and/or environmental factors that may modulate the phenotype. All the reported NKX2-1 mutations (summarized in Table 1) have variable functional effects, even if the mutations occur in similar regions of the protein. Indeed, the clinical features of the affected families and family members described in the literature are very variable, and there is no correlation between the clinical and the molecular phenotype. Mutations that produce major alterations in the protein structure may be associated with only minor neurological signs (19,26), while primary hypothyroidism has been linked to *NKX2-1* mutations in 81% of the cases (25/31) (19,20,22,27–38).

In conclusion, a novel *NKX2-1* mutation in a family presenting the clinical findings of the BTLS is described. The c.493delC mutant migrates into the nucleus but is not able to transactivate the Tg or SP-C promoters. These results indicate that NKX2-1 activity is finely regulated by the interaction of several factors that are still largely unknown. The mechanisms regulating gene expression are different in each tissue. Additional studies are required to better understand the complexities of the genotype–phenotype correlation in the NKX2-1 deficiency syndrome.

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### Disclosure Statement

All the authors in the study have no conflict of interest to declare.

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# Screening for mutations in the *ISL1* gene in patients with thyroid dysgenesis

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**ABSTRACT.** ***Context:*** **Congenital hypothyroidism (CH) is a common endocrine disorder with an incidence of 1:3000-4000 newborns. In 80-85% of cases, CH is caused by defects in thyroid organogenesis, resulting in absent, ectopically located, and/or severely reduced gland, all conditions indicated as "thyroid dysgenesis" (TD). A higher prevalence of congenital heart diseases has been documented in children with CH compared to the general population. This association suggests a possible pathogenic role of genes involved in both heart and thyroid development. Among these, it can be included *Isl1*, a transcription factor containing a LIM homeodomain that is expressed in both thyroid and heart during morphogenesis.** ***Objective:*** **In the present study, we investigate the role of *ISL1* in the pathogenesis of TD.** ***Settings and patients:*** **By single stranded conformational polymorphism, we screened for mutations the entire *ISL1* coding sequence in 96 patients with TD and in 96 normal controls.** ***Results:*** **No mutations have been found in patients and controls.** ***Conclusion:*** **Our data indicate that, despite the relevant role of *ISL1* in thyroid and heart morphogenesis, mutations in its coding region are not associated with TD in our group of patients.**

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## INTRODUCTION

Primary congenital hypothyroidism (CH) is the most frequent endocrine metabolic disease in the infancy, with an incidence of 1/3000-4000 live birth. In about 15% of cases, the disease is caused by inborn errors in the mechanisms required for thyroid hormone biosynthesis, a condition known as dysormonogenesis. In the remaining 85% of the cases, CH is caused by alterations occurring during the thyroid gland organogenesis, that results in a thyroid that is completely absent (athyreosis), severely reduced in size (hypoplasia) or located in abnormal position (ectopy). All these entities are grouped under the term of "thyroid dysgenesis" (TD) (1).

Several genes have been found mutated in patients with TD, including *NKX2.1*, *FOXE1*, *PAX8*, *TSHR*, and *NKX2.5*, suggesting that in a small percentage of cases TD is a genetic disorder (2).

Congenital malformations, mostly cardiac, have higher frequency in children with CH than in the general population (3-5), and we recently demonstrated that NKX2.5, a transcription factor that plays a crucial role in heart morphogenesis (6-8) and found altered in patients with congenital heart disease (9-12), can also be associated to TD (13). These observations suggest that genes expressed during embryogenesis of both thyroid and heart can be good candidates in the pathogenesis of TD. One of these is the gene encoding for *ISL1*, a LIM homeodomain transcription factor originally proven to be necessary for the differentiation of both exocrine and endocrine pancreas (14). Pancreatic organogenesis requires Isl1 activity in endoderm progenitors as well as in the mesenchymal tissues surrounding the dorsal pancreatic bud. A similar expression pattern of *Isl1* has been demonstrated in the anterior foregut and adjacent cardiogenic mesoderm in early steps of mouse (15) and Drosophila cardiogenesis (16). *Isl1* knock out mice show severe malformations of the heart and of the cardiac outflow tract that leads to embryonic lethality around E10.5 (15). Since thyroid progenitor cells assemble in this portion of the endoderm, it can be hypothesized that Isl1 might also play a role in this process. This possibility is also suggested by recent findings demonstrating that Isl1-regulated cardiac morphogenesis is mediated *via* Sonichedgehog (Shh) (17) and that *Shh* null mice exhibit thyroid malformations (18, 19). Finally, *Isl1* has previously reported to be expressed in the early thyroid placode in chicken (20) and in the adult rat thyroid gland, although only C-cells showed Isl1 immunoreactivity in this study (21).

Very recently, it has been showed that, in mouse embryo, *Isl1* is widely expressed in the anterior foregut endoderm including the thyroid primordia. During further developmental steps, *Isl1* expression is maintained in all thyroid progenitor cells until the anlagen fuse at 13.5. Therefore *Isl1* expression seems to be restricted to the C-cells precursors, and it is down-regulated in the presumptive follicular cells. The adult thyroid contains only few Isl1-positive cells, but their number is in clear minority compared

*Key-words:* Congenital hypothyroidism, development, hearth, thyroid dysgenesis, transcription factor.

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to the total number of C-cells. These data suggest that Isl1 is a putative transcriptional regulator of both follicular and C-cell during thyroid organogenesis (22).
Given the foregoing, we considered *ISL1* as a good candidate gene for TD. In the present work, *ISL1* mutational screening was carried out in a group of 96 patients with TD but no mutation has been identified.

## MATERIALS AND METHODS

### *Patient recruitments*

Ninety-six patients with permanent CH (35 with ectopy, 31 with hypoplasia, 30 with athyreosis), were included in the study. For all of them, together with blood sample, pediatricians provide a questionnaire with several clinical informations. With the exception of 3 cases, no congenital heart defects have been indicated in the questionnaire, suggesting that patients did not presented relevant cardiac alterations. In 3 patients minor heart congenital defects have been reported: 2 patients (1 with thyroid ectopy and the other 1 with thyroid hypoplasia) with patent foramen ovale at birth that resolved spontaneously and 1 (with thyroid ectopy) with minor pulmonary valve insufficiency. Screening for mutations in other genes associated to TD (*NKX2.1*, *FOXE1*, *PAX8*, *TSHR*) and in *NKX2.5* (associated with TD and congenital heart disease) was negative in all the patients. Also 96 healthy subjects with no thyroid-related disorders were also enrolled as controls. The study was approved by the Ethics Committees of the participating Institutes. All individuals, or parents of minors, gave their informed consent.

### *Single stranded conformational polymorphism*

The entire *ISL1* coding region was screened for mutations on genomic DNA extracted from peripheral blood lymphocytes. Oligonucleotide primers (sequence available upon request) were designed on the human *ISL1* cDNA (NM_002202) and genomic sequences to produce PCR products suitable for single stranded conformational polymorphism (SSCP) analysis (Fig. 1). PCR reactions were carried out in 10 µl reaction volume with the following cycling parameters: 94 C, 8 min; 94 C, 45 sec; the specific temperature for each exon, 30 sec; 72 C, 45 sec for 35 cycles; 72 C, 15 min. Samples were denatured 1:1 in denaturing solution (99% formamide, 1% xylene cyanol solution, 0.1% bromophenol blue) at 95 C for 5 min and thereafter directly placed on ice to prevent reannealing of the single stranded product. Six µl of each sample were applied to a nondenaturing gel of acrylamide (GeneGel TM Excel 12.5/24 Kit, GE Healthcare Bio-Sciences AB, Sweden) and run on a GenePhorTM Electrophoresis Unit (GE Healthcare Bio-Sciences AB, Sweden). The running conditions were the following: plate temperature: 15 C, 600 V, 25 mA, 15 W. The running was stopped when bromophenol blue reached the anode buffer strip (at least 80 min). Following separation, we stained nucleic acids in acrylamide gel using the PlusOneTM DNA Silver Staining Kit (GE Healthcare Bio-Sciences AB, Sweden), following producer's protocol.

### *Direct sequencing*

PCR products of the samples showing an abnormal migration at the SSCP were purified with Antartic Phosphatase-Exonuclease I (New England BioLabs, USA) at the following conditions: 37 C for 15 min and 80 C for 15 min, respectively for enzymes activation and inactivation, and then, directly sequenced bi-directionally with a 377 ABI sequencer.

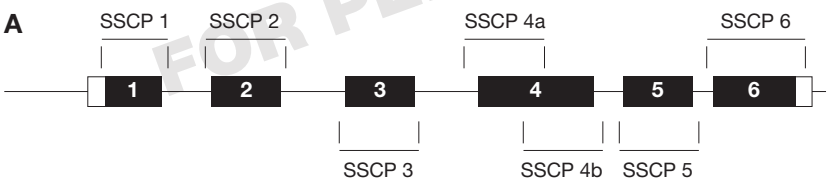
### *Statistical analysis*

Statistical analysis were performed using the 2×2 contingency table available on line at http://graphpad.com/quickcalcs/contingency1.cfm. *p* values were calculated using the Fisher's exact test.

## RESULTS

SSCP method was used as first approach to screen for mutations the *ISL1* coding sequence in patients with TD using specific primer-pairs. Examples of the SSCP are






**B**

| SSCP | PCR length | Annealing T |
|---|---|---|
| 1 | 337 bp | 59 C |
| 2 | 287 bp | |
| 3 | 367 bp | |
| 4a | 338 bp | |
| 4b | 248 bp | |
| 5 | 228 bp | |
| 6 | 235 bp | 51.2 C |

**C**

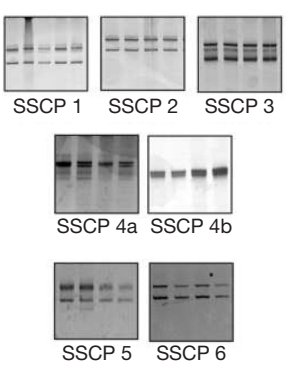



Fig. 1 - *Panel A shows a schematic representation of the human WWTR1 gene and the location of the primers used for single stranded conformational polymorphism (SSCP) analysis. In panel B, the PCR product length and the annealing temperature of the respective PCR amplification are reported for each DNA exon. In panel C, typical results of SSCP analysis are shown. Please note in SSCP4a the different migration patterns of samples 1 and 3 compared to samples 2 and 4. They correspond to a heterozygous CCA to CCG change in the triplet encoding for proline at position 168 of ISL1.*

shown in Figure 1. No mutations have been identified in DNA extracted from either patients or controls. We found a silent polymorphism corresponding in a CCA to CCG change in the triplet encoding for proline at position 168 in 8 patients (4 with ectopy and 4 with hypoplasia) in heterozygous state and in 10 patients (5 with athyreosis, 2 ectopy, 1 with thyroid ectopy and patent foramen ovale and 2 with CH not further characterized) in homozygous state. The same polymorphism was found in 28 controls (8 in heterozygous state and 20 in homozygous state). This polymorphysm has been previously reported (http://www.genecards.org). Statistical analysis (Fisher's exact test) demonstrated no significant difference between patients and controls.

## DISCUSSION

The genetic origin of TD is still wildly discussed, despite several genetic factors have been demonstrated as cause of CH in animal models and in a small percentage of patients with TD.

Since TD is frequently associated to other congenital malformations, mostly cardiac (3-5), it is possible to hypothesize that genes involved in heart organogenesis might also participate to thyroid development. The ventral pharyngeal endoderm, from which the thyroid follicular cells originate, lies in close opposition to the heart mesoderm, and the role of the endoderm in the "specification" and differentiation of myocardial cells has been already defined (23). Conversely, the influence of the developing heart on thyroid organogenesis has not been demonstrated, although a recent work identify a critical role of developing arteries on thyroid development (18). The hypothesis of close relationship between developing heart and thyroid is also confirmed by the recent identification of mutation in the NKX2.5 gene, a transcription factor critical for the heart morphogenesis, in patients with TD (13). Based on these findings, we are investigating the possible role of genes involved in heart development for their role in thyroid organogenesis, and *ISL1* could be considered an attractive candidate in the pathogenesis of TD.

Cai et al. showed that *Isl1* null mice die at approximately E10.5 because of severe cardiovascular malformations. These are determined by the lack of Isl1-expressing cardiogenic progenitor cells in embryonic heart and as consequence of a decreased cell proliferation and increased apoptosis in the endoderm and in the adjacent splanchnic mesoderm in *Isl1* null mice embryos (15).

More recently Wendl et al. demonstrated a role for cardiac mesoderm in early thyroid development (24): thyroid progenitor cells were missing and the thyroid failed to develop in zebrafish mutants deficient of Hand2, a transcriptional factor involved in heart development. Since Hand2 was suggested to function downstream of Isl1 in the developing heart (25), it might be possible that the Isl1-dependent signals in the pharyngeal mesoderm might also influence the growth of the thyroid bud.

Westerlund et al. speculated that *Isl1* might be an interesting gene involved in the control of the early phases thyroid formation (thyroid specification), and demonstrated that Nkx2.1-positive thyroid progenitor cells express *Isl1* during evagination from the pharyngeal endoderm at E10.75 and when the thyroid is completely detached at E11.5 (22). Moreover, *Isl1* is expressed in the endoderm of the 4$^{th}$ pouches at E10.75 and in the completely detached E12.5 ultimobranchial bodies, from which calcitonin-producing cells (C-cells) originate. At E13, *Isl1* expression is present in the trachea, in the ultimobrachial bodies, in midline thyroid primordium, and in the mesenchyme surrounding the trachea, but, after the fusion of the cells deriving from the ultimobrachial bodies with the midline precursors of the follicular thyroid cells at E13.5, *Isl1* is present only in the thyroid, while its expression is markedly decreased in the midline mesoderm and trachea. *Isl1* expression disappears when the number of C-cells gradually increase between E15.5 and E17.5 (22). Of note, starting from E9.5 the thyroid placode is significantly smaller in *Isl1*$^{-/-}$ mice when compared wild type embryos, and the thyroid follicular cells at this stage present a normal *Nkx2.1* and *Pax8* expression (22). Finally, in cardiac progenitors *Nkx2.5* is transcriptionally regulated by Isl1 (26). Takeuchi and coworkers studied the upstream regulatory regions of the proximal *Nkx2-5* enhancer and identified a conserved Isl1-binding site, adjacent to the Gata site required for *NKX2.5* expression in the anterior heart field. Mutations in this Isl1-binding site abolish Nkx2.5 expression in the right ventricle and in the outflow tract. We previously demonstrated the association between NKX2.5 mutation and thyroid dysgenesis (13), and Takeuchi's data clarify the strong interaction between these two transcription factors, at least in the developing heart.

All these observations allow to hypothesize a possible role of ISL-1 also in the thyroid development. In the present study, the entire coding region of the *ISL1* gene was screened for mutations in patients with CH due to thyroid dysgenesis in order to evaluate the potential role of this gene in the pathogenesis of TD. We were not able to demonstrate mutations in the *ISL1* coding sequence in 96 patients with TD as well as in 96 healthy normal controls.

The absence of *ISL1* mutations in our patients well correlate with the complex phenotype observed in *Isl1* knock-out mice, were the severe cardiac malformations are responsible for early embryonic lethality (22), and it is possible to hypothesize that alterations in this gene are not compatible with life also in humans as well as in mice. Our results do not exclude the potential role of *ISL1* in the pathogenesis of TD, since we have not investigated other genes potentially necessary for ISL1 activity, including cofactors or cell-specific co-activators. Moreover, mutation(s) in the *ISL1* promoter responsible for a decrease in the expression of the gene, would have been undetected in this study as well as mutations occurring in intronic regions of the gene that can produce inactive splice variants.

In conclusion, despite the fact that *ISL1* was a good candidate for TD, we did not find mutations in the coding region of this gene in patients with TD alone as well as in the 3 patients with TD and congenital heart disease. Our results could be explained by the essential role of this factor in embryonic heart development. As described in mouse models, it should be hypothesized that patients with *ISL1* mutations die before birth because of heart ma-

jor defects. In these patients TD might be not diagnosed and the detection of *ISL1* mutations lost.

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