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T3/TRβ$_1$ induces proliferative effects on papillary thyroid cancer cells FB2

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INTRODUCTION

A number of clinical and experimental studies have suggested the crucial role of thyroid hormones (THs) and cognate nuclear receptors in cell growth and differentiation of many cell types (*Pibiri et al., 2001, Bassett and Williams, 2003, Jones et al., 2003, Lin et al., 2009, Verga Falazacappa et al., 2009*). Although recently some authors have demonstrated the involvement of TH in cell proliferation of many tumor cell types, including thyroid cancer cell lines, the exact mechanisms through which TH induces tumor cell proliferation still is not well understood (*H.Y.Tang et. al 2004, M.Cristofanilli et. al 2005 , F.B.Davis et. al 2006*; *G.B.Hernandez et. al 1999, F.B.Davis et. al 2006, H.Y.Tang et. al 2004*; *M.L.Hsieh and H.H.Juang 2005*; *P. Poplawski et al 2008).*

The actions of TH occur through its binding to the thyroid hormone receptors (TRs): TRα and TRβ that mapped to human chromosomes 17 and 3 respectively *(Harvey et al., 2002).* The two genes, TRα and TRβ, encode several major isoforms: $TR\alpha_1$, $TR\beta_1$, $TR\beta_2$, which bind T3 with similar affinity and have similar transcriptional activity. The TRα gene encodes two different proteins: $TR\alpha_1$ and c-erbAα_2, that are generated by alternative-splicing of TRα mRNA. Of note, c-erbAα_2 cannot bind T3 nor transcriptionally regulate target genes because it contains a 122-amino-acid carboxy-terminus, which replaces a sub-region in the $TR\alpha_1$. The TRβ gene encodes two major identical TRβ isoforms: $TR\beta_1$ and $TR\beta_2$. Both $TR\alpha_1$ and $TR\beta_1$ mRNAs and proteins are expressed in almost all tissue.

TRs share structural and functional similarities with other members of the nuclear receptor superfamily such as those for adrenal steroids, sex hormones, vitamin D and retinoic acid *(Evans, 1988).* Nuclear receptors possess a well-conserved DNA binding domain (DBD) separated from a carboxy-terminal ligand binding domain

(LBD) by a short segment of amino acids that constitutes the “hinge” region. The DBD of TRs contains two stretches of 13 and 12 amino acids separated by pairs of cysteines that interact with zinc to create two peptide loops *(Evans, 1988)* (Fig. 1).

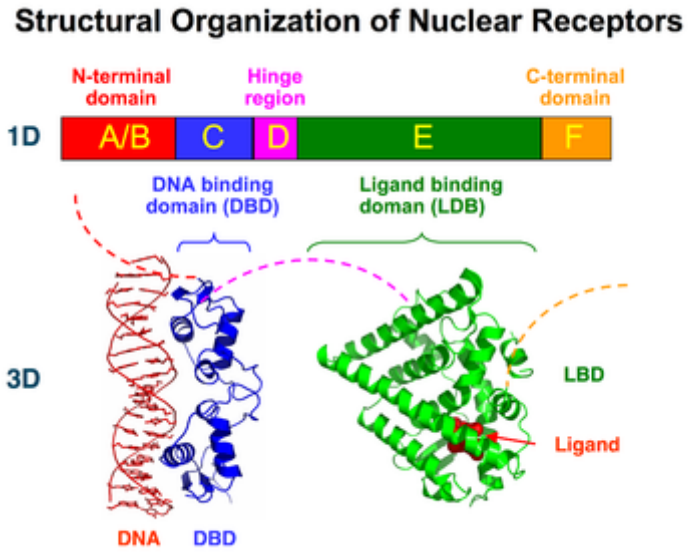


Fig. 1. Organization of major thyroid hormone receptors (TR) domains

These “zinc-fingers”, projecting from the surface of the protein, interact with specific DNA sequences known as TH response elements (TREs) located usually near the transcription start point of genes regulated by TH. Transactivation of these target genes requires activation of the receptor by hormone binding to the LBD and the presence of additional cofactors. A highly conserved region in the distal carboxy-teminal of the LBD, termed activation function-2 (AF-2), has a little effect on ligand binding or dimerization. AF-2, however, is necessary for nuclear coactivator (NCoA) transcriptional activation because it is composed of an amphipathic alpha-helix that interacts with NCoAs *(Feng et al, 1998; Tone et al, 1998).*

TRs bind to TRE that are typically located in the upstream promoter regions of target genes. In positively regulated genes, TRE generally contain two or more hexamer half-site sequences of AGGT(C/A)A arranged in tandem array. Generally, TRs can bind to TREs in which half-sites are arranged as direct repeats inverted palindromes, and palindromes that contain optimal spacings of four, six, or zero nucleotides between half-sites, respectively. It is likely heterodimerization with RXR enables TR to bind to a wide repertoire of nucleotide sequences and motifs.

In addition to TR-mediated transcriptional action by TRs, non-transcriptional pathways are regulated by THs. Evidence for these non-genomic effects include the lack of dependence on nuclear TRs, structure-function relationships of TH analogs that are different than their affinities for TRs, rapid onset of action (typically seconds to minutes), occurrence in the face of transcriptional blockade and utilization of membrane-signaling pathways. Some of these effects involve TR, particularly TR located outside the nucleus, whereas others utilize other proteins that can bind TH, such as the integrin αVβ3.

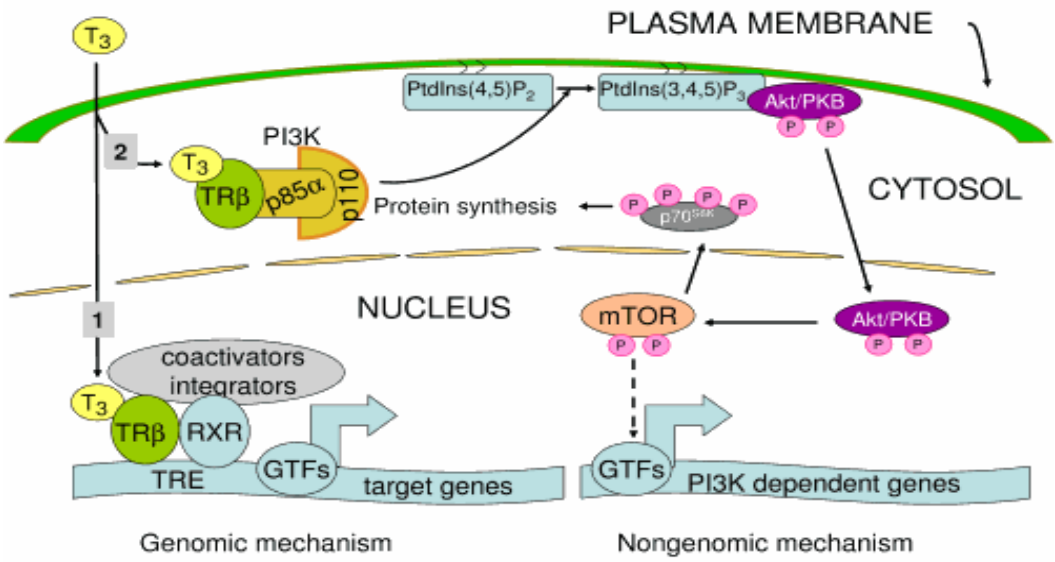


Fig. 2. Genomic and non-genomic action of TH

Recently, Davis and colleagues identified integrin αVβ3 as a plasma membrane TH-binding site. Previously, they showed that both T4 and T3 activated mitogen-activated-protein kinase (MAPK) activity, leading to serine phosphorylation of $TR\beta_1$ as well as to $TR\beta_1$ traslocation into the nucleus and co-repressor release. Purified radiolabeled T4 and T3 bind specifically to integrin αVβ3. Moreover, siRNAs against the integrin αVβ3 subunits block MAPK activation by TH in CV-1 cells. These data thus provide strong evidence that TH activates the MAPK cascade via TH binding to a membrane receptor, integrin αVβ3. This mechanism leads to phosphorylation of nuclear receptors and can induce angiogenesis and promote cell growth *(Oetting, 2007; Davis et al., 2007)* (Fig. 3).

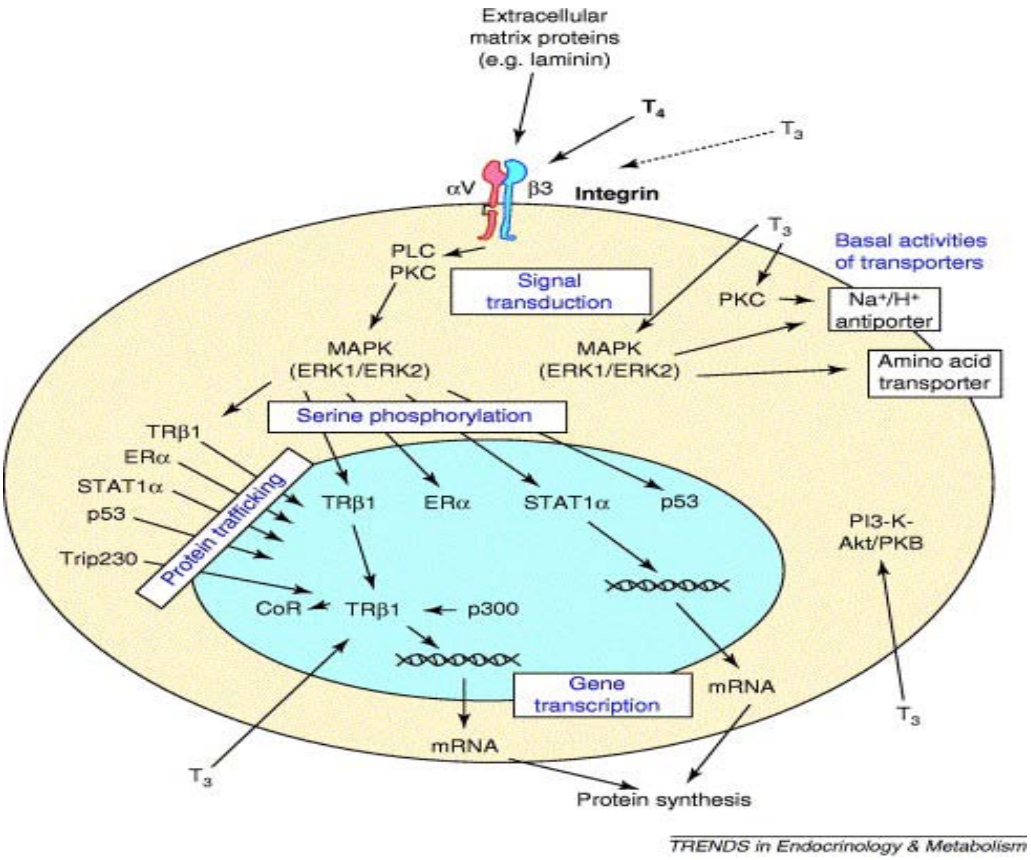


Fig. 3. Summary of membrane-initiated cellular actions of thyroid hormone

Many laboratories have reported tumor cell proliferation in vitro in response to TH and there are also several reports of anti-apoptotic actions of the hormone on specific tumor cell lines. Davis et al. have demonstrated that TH, acting at integrin αVβ3 receptor, non-genomically activates MAPK signal transduction cascade, causing proliferation of human papillary and follicular thyroid cancer cell line. Moreover they showed that TH was able to exert an anti-apoptotic effect in both thyroid cancer cell lines *(Davis et al., 2006).*

In this study we investigated if T3 non-genomic signaling, was able to stimulate cell proliferation and cell survival in a well-differentiated papillary thyroid cancer cell line, designed FB2.

MATERIALS AND METHODS

Reagents

3,5,3'-Tri-iodothyronine (T3), RDG-peptide, PD-98059 and LY-294002 hydrochloride were obtained from Sigma-Aldrich (St Louis, MO, USA)

Cell Culture

Human thyroid papillary carcinoma cell line (FB-2) were established and characterized by Basolo et al. FB-2 cells, derived from a well-differentiated papillary carcinoma, harbor the RET/PTC1 chimeric oncogene in which the RET kinase domains is fused to the H4 gene. FB-2 cells only partially retained the differentiated thyroid phenotype. In fact, the PX-8 gene, which codes for a transcriptional factor required for thyroid cell differentiation, was expressed, while tyreoglobulin, TSH-receptor and TPO genes were not. Moreover, FB-2 cells produced high levels of interleukin (IL-6) and IL-8 *(Basolo et al., 2002).*

FB-2 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) plus glutamax, (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 1 mg/ml penicillin-streptomycin (P/S). Cells were maintained at 37°C under humidified conditions of 95% air and 5% CO2.

FB-2 were cultured to 60% confluence and exposed to treatments added only once, at the beginning of the individual experiments. Prior to treatments, cells were harvested for 24 hours.

Human Follicular Thyroid cancer cells WRO (a gift from Dr Arturi, University of Magna Grecia, Catanzaro, Italy) were grown in Dulbecco's Modified Eagle's medium (DMEM) plus glutamax containing 10% fetal bovine serum (FBS, Invitrogen) and 1 mg/ml penicillin-streptomycin (P/S).

Proliferation assays

Cell proliferation by (^{3}H)thymidine incorporation

FB-2 cells were seeded in 6-well plates in a regular growth medium. On the second day, the cells were incubated in serum free medium (SFM) for 24 hours and then cultured with treatments. (^{3}H)thymidine (1 μCi/ml; New England Nuclear, Newton, MA, USA) was added to the medium for last 6 hours of the second day. After rinsing with PBS, the cells were washed once with 10% and thrice with 5% trichloroacetic acid. The cells were lysed by adding 0,1 M NaOH and then incubated for 30 min at 37°C. Thymidine incorporation was determinate by scintillation counting.

Reverse transcription-PCR (RT-PCR) assay

Cells were grown in 10 cm dishes to 60-70% confluence and exposed to treatments for 24 h in SFM. The total cellular RNA was extracted using TRIZOL reagent (Invitrogen) as suggested by the manufacturer. The purity and integrity were checked spettroscopically and by gel electrophoresis before carrying out the analytical procedures. The evaluation of gene expression was perfomed by the semiquantitative RT-PCR method. For TRβ_1, TRα_1 and the internal control gene 36B4, the primers were: 36B4 forward: 5'-CTC AAC ATC TCC CCC TTC TC-3'; 36B4 reverse: 5'-CAA ATC

CCA TAT CCT CGT CC-3'; TRα1 forward: 5'-GCC AAA AAA CTG CCC ATG TTC TCC GAG-3'; TRα1 reverse: 5'-GGC AGG CCC CGA TCA TGC GGA GGT CAG-3'; TRβ1 forward: 5'-CTC TGT GTA GTG TGT GGT GA-3'; TRβ1 reverse: 5'-TCA TCC AGC ACC AAA TCT GT-3' to yield respectively the products of 408 bp with 18 cycles, 445bp with 25 cycles and 229bp with 40 cycles.

Immunoblotting

The cells were grown in 10 cm dishes to 70-80% confluence and exposed to treatments in SFM, as indicated. They were the harvested in cold PBS and resuspended in a lysis buffer (RIPA-buffer) containing 50 mM Tris-HCl, (pH 7.5), 150 mM NaCl 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 1% Sodio-Dodecil-Solfato (SDS) and inhibitors (0,1mM Na3VO4, 1% phenylmethylsulphonyl fluoride (PMSF), 20mg/ml aprotinin).

The protein concentration was determinated using Bio-Rad Assay (Bio-Rad Laboratories). A 50µg portion of protein lysates was used for western blotting (WB), resolved on a 10% SDS-polycrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). Filters were blocked for non-specific reactivity by incubation for 1 h at RT in 5% non-fat dry milk dissolved in TBST 1X and then incubated for 16 h at 4°C with: $TR\beta_1$ (Santa Cruz Biotechnology), Total Akt (Santa Cruz), Total ERK (Santa Cruz), phospho Akt 1/2/3-Ser 473 (Santa Cruz), phospho ERK 1/2 (Santa Cruz). As loading, all membranes were subsequenthly stripped of the first antibody and reprobed with anti-GAPDH antibody (Santa Cruz), anti-β-actin antibody (Santa Cruz), anti-Lamin B antibody (Santa Cruz). The antigen-antibody complex was detected by incubation of the membranes for 1 h at RT with peroxidase-coupled goat anti-mouse or anti-rabbit IgG

and revealed using the enhanced chemiluminescence system (ECL system, Amersham Pharmacia). The blots were then exposed to Kodak film (Sigma).

Immunoprecipitation

Cells were lysed as previously described; cell lysate (500µg) was incubated for 2 h with 20 µL protein A/G-agarose beads at 4 C and then centrifuged at 12,000 X *g* for 5min. The supernatants were then incubated overnight with 10 µl mouse anti- $TR\beta_1$ (Santa Cruz, 1 µg) and 20 µL of protein A/G. Immunoprecipitates were collected by centrifugation at 12,000 X g for 10 minutes, followed by washing three times with HNTG (IP) buffer (50 mm HEPES, pH 7.4; 50 mm NaCl; 0.1% Triton X-100; 10% glycerol; 1 mm phenylmethylsulfonylfluoride;10 µg/ml leupeptin; 10 µg/ml aprotinin; 2 µg/ml pepstatin). Following the final wash, supernatant was removed. Samples were resuspended in the Laemmli sample buffer, subjected to SDS-polyacrylammide gel electrophoresis (10% gel) and then transferred onto a nitrocellulose membrane. The immunoprecipitated proteins were detected by Western Blot using a rabbit anti-PI3K p85α (Santa Cruz, 1:500) and mouse anti- $TR\beta_1$. Immunoprecipitation with protein A/G alone was used as negative control. Membranes were stripped of bound antibodies by incubation in glycine (0.2 m, pH 2.6) for 30 min at room temperature. Before reprobing with different primary antibodies, stripped membranes were washed extensively in TBS-T and placed in blocking buffer (TBS-T containing 5% milk) overnight.

PI3K Kinase assay

Cells were grown in 10 cm dishes to 70-80% confluence and exposed to treatments for 15 minutes in SFM and then lysates with 500µL of lysis buffer (50 mm HEPES, pH 7,5; 150 mmmol/L NaCl, 1,5 mmol/L MgCl2, 1mmol/L EGTA, 10% glycerol, 1% Triton X-

100, a mixture of protease inhibitors ((0,1mM Na3VO4, 1% phenylmethylsulphonyl fluoride (PMSF), 20mg/ml aprotinin)). Cell lysates were centrifuged at 12,000 x g for 5 minutes and 500µg of total protein were incubated overnight with the anti-p85α antibody (Santa Cruz Biotechnology) and 500µL of HNTG (immunoprecipitation) buffer (50 mmol/L HEPES, pH 7,5; 50 mmol/L NaCl, 10% glycerol, Triton X-100, 1% phenylmethylsulphonyl fluoride, 10mcg/ml leupeptin, 10mcg/ml aprotinin, 2mcg/ml pepstatin. Immunocomplexes were recovered by incubation with protein A/G-agarose. The immunoprecipitates were washed once with cold PBS, twice with 0,5 M LiCl, 0,1 M Tris (pH=7,4) and finally with 10mM Tris, 100mM NaCl and 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mm HEPES (pH 7.4), 10 mm MgCl2, 50 µm ATP, 20 µCi [γ-^{32}P] ATP and 10 µg L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P2) for 20min at 37 C. The reactions were stopped by adding 100 µl of 1 m HCl. Phospholipids were extracted with 200 µl CHCl3/methanol. For extraction of lipids, 200 µl chloroform:methanol (1:1, vol/vol) were added to the samples and vortexed for 20 sec. Phase separation was facilitated by centrifugation at 5000 rpm for 2 min in a tabletop centrifuge. The upperphase was removed, and the lower chloroform phase was washed once more with clear upper phase. The washed chloroform phase was dried under a stream of nitrogen gas and redissolved in 30 µl chloroform. The labeled products of the kinase reaction, the PI phosphates, then were spotted onto *trans*-1,2-diaminocyclohexane-*N*,*N*,*N*_,*N*_-tetraacetic acid–treated silica gel 60 thin-layer chromatography plates state running solvent used for TLC. Radioactive spots were visualized by autoradiography.

RNA interference (Rnai)

Cells were plated in 10 cm dishes in the regular growth medium the day before transfection to 60-70% confluence. On the second day, the medium was changed with SFM without P/S and the cells were transfected with 25bp RNA duplex of validated RNAi targeted human $TR\beta_1$ mRNA sequence 5'UUGAUGAGCUCCCAUUCCUCGUCUG3'sequence or with a stealth RNAi control (Invitrogen) to a final concentration of 100nM, using Lipofectamine 2000 (Invitrogen), as recommended by the manufacturer. After 5 h, the trasfection medium was changed with SFM in order to avoid Lipofectamine toxicity and the cells were exposed to T3 and then treated for WB analysis and proliferation assay.

Immunofluorescent microscopy

50% confluent cultures, grown on coverslips, were shifted to SFM for 24 h and then treated either for 20 minutes with T3 (10^{-7}M). Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 h with primary Abs recognizing $TR\beta_1$ (Santa Cruz Biotechnology). The anti- $TR\beta_1$ monoclonal Ab (mAb) (Santa Cruz) at 2 mg/ml was used for $TR\beta_1$ staining. Following the incubation with primary Abs, the slides were washed three times with PBS, and incubated with a secondary Abs, each 1 mg/ml concentrated. A rhodamine-conjugated donkey anti-mouse IgG (Sigma) was used as a secondary Ab for $TR\beta_1$. The cellular localization of $TR\beta_1$ was studied with fluorescence microscope with 1000 × magnification. The optical sections were taken at the central plane.

Statistical analysis

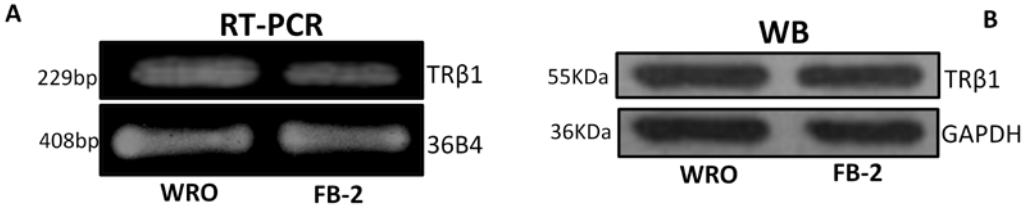
Statistical analysis was performed using ANOVA followed by Newman-Keuls testing to determine differences in means. $p<0,05$ was considered as statistically significant.

RESULTS

Thyroid hormone receptor β_1 is expressed in FB-2 cells

First of all we ascertained the expression of the two major isoforms of TR in FB-2 cells, TRα_1 and TRβ_1, to demonstrate that the effects of T3 on FB2 cells were specifically mediated by the binding of thyroid hormone to its own receptor.

As showed in Fig. 1A, by RT-PCR, TRβ_1 mRNA was detected in FB-2 cells and similar results were observed by Western Blot analysis (Fig. 1B). Follicular thyroid cancer cells WRO were used as positive control. In contrast, very low levels of TRα_1 mRNA was revealed, while TRα_1 protein was undetectable (data non shown). These findings indicated that TRβ_1 is the predominant TR isoform in FB-2 cells. Previous studies reported that TRβ_1 receptor is present at cytoplasmatic and nuclear levels *(Davis et al., 2000; Zhu et al., 1998).* Thus, in our cellular context, we performed immunostaning assay to identify the localization of the thyroid hormone receptor β_1 in the cell compartment. Our results showed that TRβ_1 receptor was clearly detectable both at the cytoplasmatic and at nuclear level, confirming its typical localization and that its expression was not affected by the rapid TH treatment (Fig.1C).



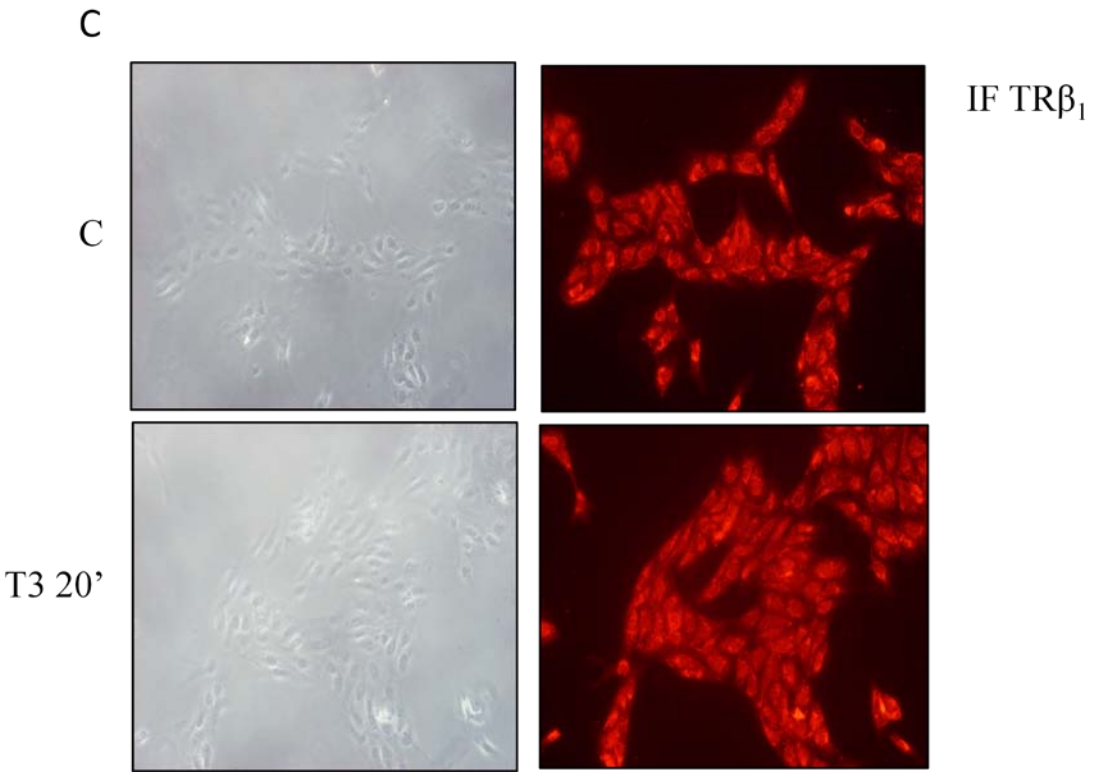


Fig. 1. Expression of $TR\beta_1$ in two human thyroid cancer cell line: WRO and FB-2 (A) RT-PCR analysis showing the expression of mRNA levels of $TR\beta_1$ in WRO and FB-2 cells. 36B4 gene served as an internal control. (B) Western Blotting analysis using a $TR\beta_1$-specific antibody. GAPDH protein served as loading control. (C) $TR\beta_1$ evaluation by fluorescence microscopy in FB2 cells fixed, permeabilized and stained with anti-$TR\beta_1$antibody. Cells were treated for 20 minutes with T3 (10^{-7}M). Data are representative of three independent experiments.

Thyroid hormone T3 induces a proliferative effect in human papillary thyroid cancer FB-2 cell line

Previous studies have demonstrated the ability of T3 to exert a proliferative effects in several tumor cells lines *(Hsieh et al., 2005;Davis et al., 2006; Lie et al., 2006; Hall et al., 2008; Poplawski et al., 2008; Lin et al., 2009).* Thus, we aimed to investigate if T3 was able to induce a proliferative effect in a new human thyroid cancer cell line, designated FB-2, that was derived from a well-differentiated papillary carcinoma. To this aim, FB-2 cells were treated with increasing doses of T3 for 48 hours. As showed in Fig. 2, T3 induced cell proliferation in a dose-dependent manner. Notably, the proliferative effect was yet significant using T3 at 1nM concentration, with higher increment at 100nM; moreover, we observed that the proliferative effects persisted after 96 hours. These results demonstrate the ability of T3 to stimulate growth of papillary thyroid cancer cells in vitro.

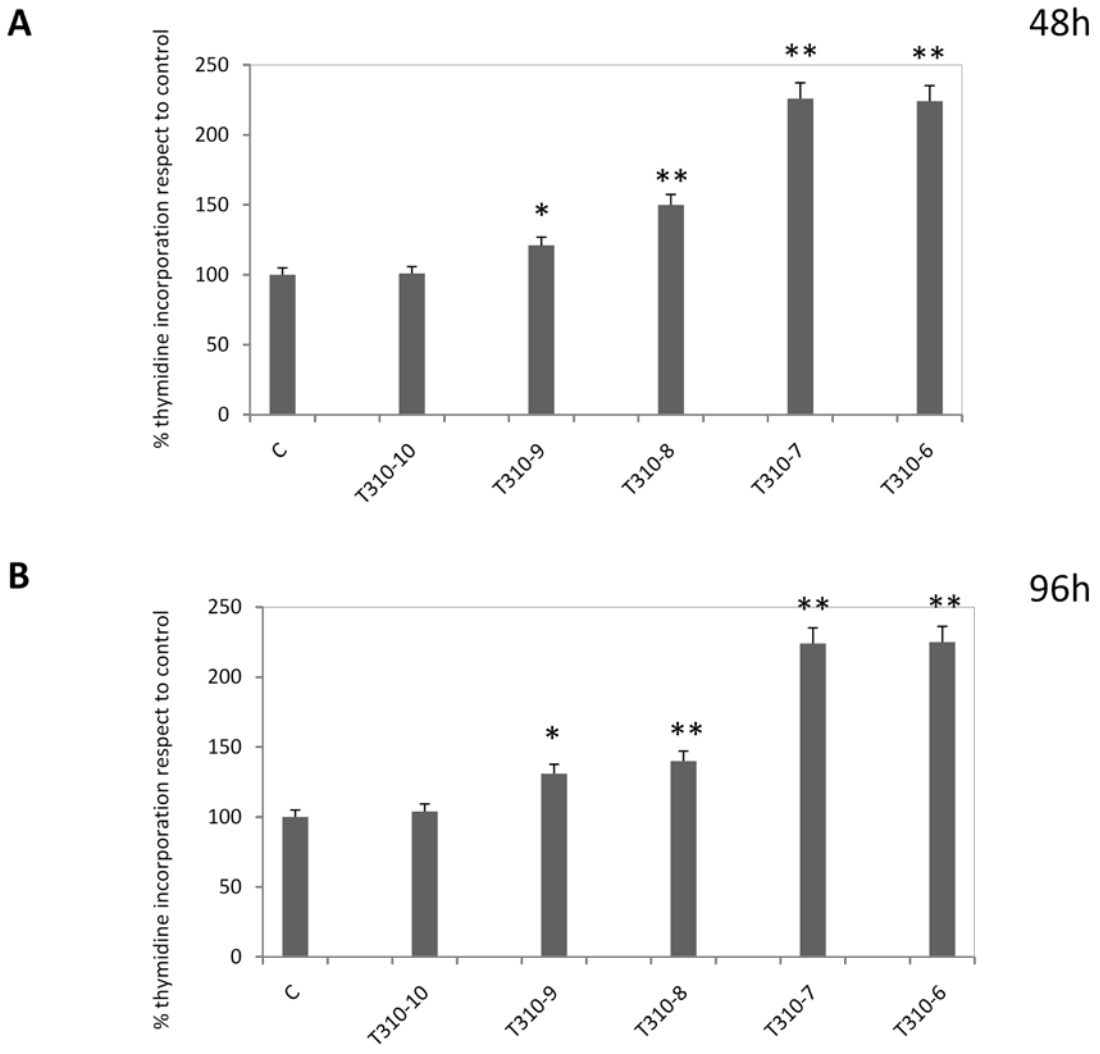


Fig. 2. Proliferative effect exerted by T3 in FB-2 cells. Cells were cultured in the presence of increasing doses of T3. Six hours before lysis (^{3}H)thymidine incorporation was added. The results represent the means ± SD of three independent experiments, each performed with triplicate samples and expressed as percentage of growth vs control which was assumed to be 100%. Statistical significance is shown as * $p<0,01$; ** $p<0,05$ vs control.

TRβ$_1$ mediates T3 action on FB-2 cells proliferation

Previous studies demonstrated that TRβ$_1$ is essential for the T3 action on the hCM cell proliferation, survival and size *(Verga Falzacappa et al., 2006).* To explore the involvement of thyroid hormone receptor β$_1$ in the proliferative effect induced by T3 in FB-2 cells, we performed proliferative assay after knoking down TRβ$_1$ with a specific siRNA. Our data showed that the cell growth effect exerted by T3 was reduced in the TRβ$_1$ silenced cells, while no changes was observed after transfection of cells with scrambled RNA upon identical experimental condition (Fig.3A). These results demonstrates the crucial role of this receptor in the T3 induced FB-2 cells proliferation.

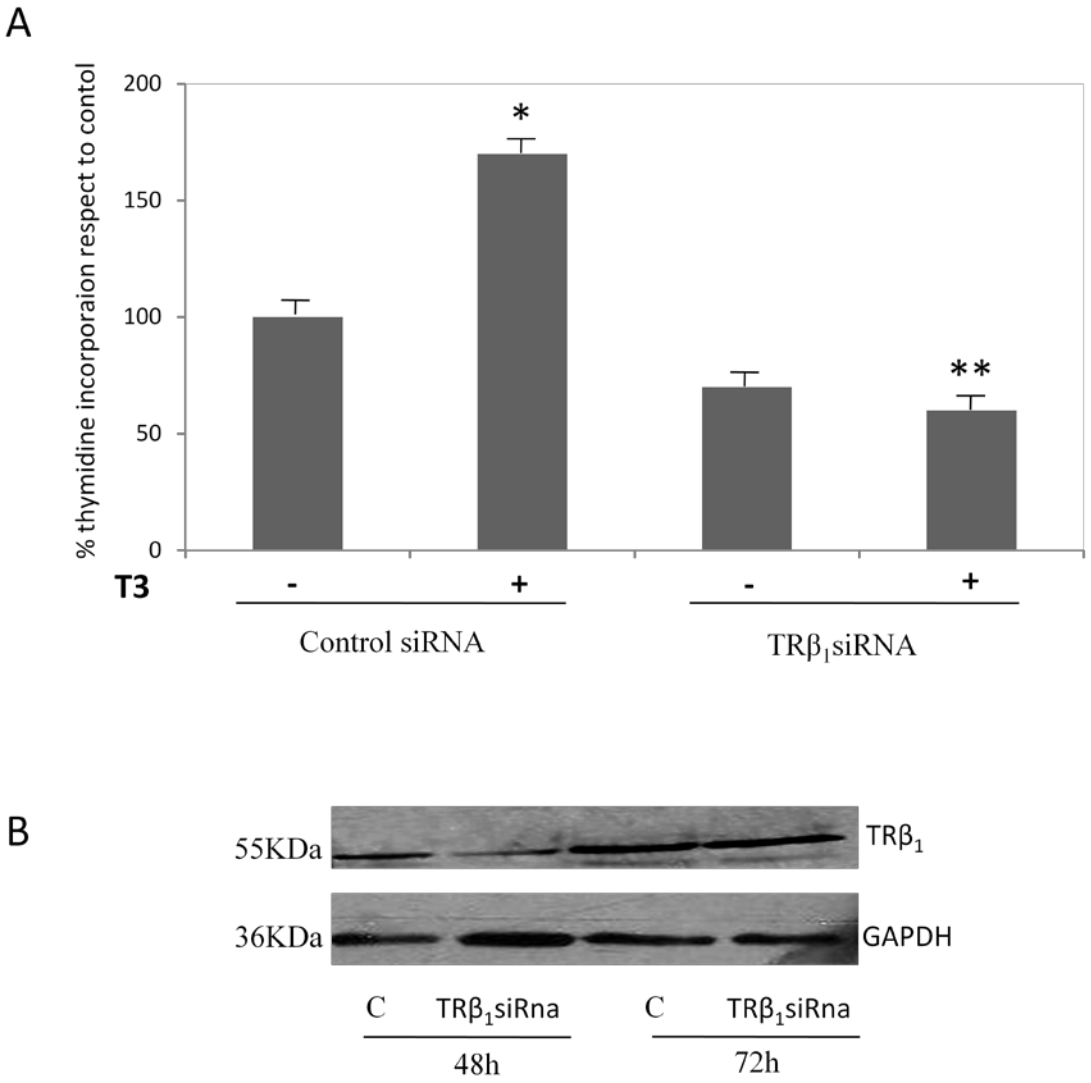


Fig. 3 $TR\beta_1$ mediates T3 action on FB-2 cells proliferation. (A) RNA interference experiments to silence $TR\beta_1$ expression were performed as described in Materials and methods. FB-2 cells were treated for 48 h with T3 (10^{-7} M). After 48 hours, the proliferative effect was determined using (^{3}H)thymidine incorporation. Data represent the mean ± SD of three independent experiments performed in triplicate and expressed as percentage of growth vs control which was assumed to be 100%. *p< 0,05 treated vs control ** <0,05 vs T3-treated in absence of RNAi$TR\beta_1$. (B) $TR\beta_1$ protein expression (evaluated by WB) in FB2 cells transfected with RNA interference targeted human $TR\beta_1$ sequence. GAPDH was used as loading control.

$TR\beta_1$ receptor mediates the rapid activation of AKT induced by T3 in FB2 cells

Considering the proliferative effect induced by T3 in FB-2 cell, we addressed the contributions of both ERK1/2 and PI3K signal transduction pathways in the action of T3 to specifically assess whether the cell proliferation effects of hormone utilize these pathways. To this aim we decided to examine an earlier time course of ERK1/2 and Akt activation by T3 treatment. Cells were cultured in the presence or the absence of the hormone treatment (T3 10^{-7}M) for 10 and 30 minutes. As shown in Fig 4A, a significant increase in Ser 473 phosphorylation of Akt and pERK1/2 was detected as early as 10 minutes after T3 addition and persisted for up 30 minutes.

In the presence of $TR\beta_1$ knocked down, we observed a significant reduction in the phosphorylation of Akt T3-induced (Fig. 4B). Notably, the activation of MAPK induced by hormone treatment still persisted in the presence of $TR\beta_1$ siRNAs. This latter data indicate that T3 membrane signaling, different from those mediated by $TR\beta_{1,}$ may be responsible for the maintainement of MAPK stimulation. For instance, it has been identified on the extracellular domain of integrin αVβ3 a cell surface receptor for thyroid hormone which activation induces cellular MAPK signal transduction cascade. Since thyroid hormone and integrin interaction occurs at or near the Arg-Gly-Asp (RGD) peptide sequence, we used an RGD peptide to verify if displacement of thyroid hormone from integrin could block thyroid hormone-MAPK-activation. As shown in Fig 4C, after 3 hours, the RGD treatment was able to reverse the activation of ERK1/2 by T3 (Fig 4C).

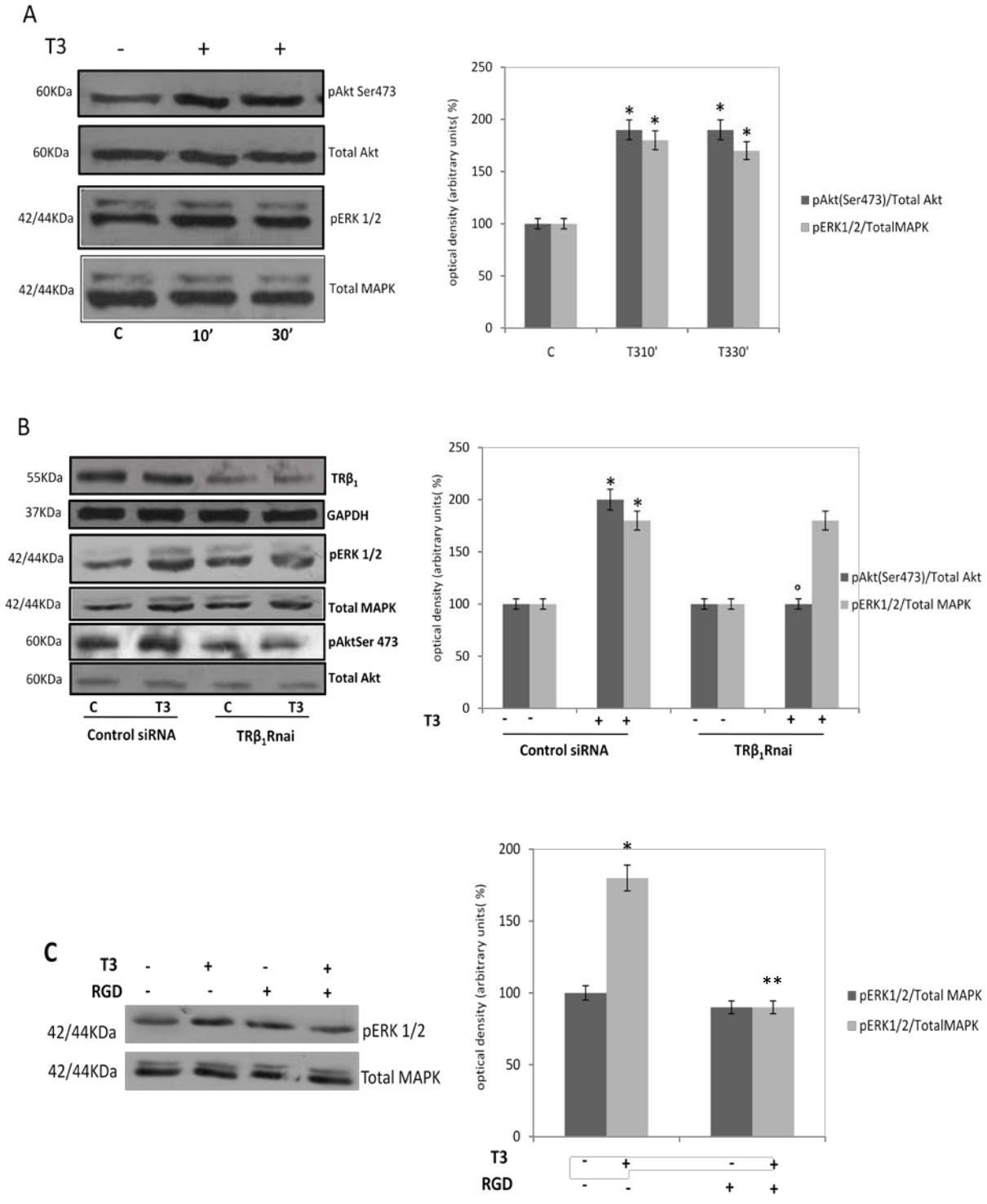


Fig. 3 TRβ_1 receptor mediates the rapid activation of AKT induced by T3 (A) Before the treatment, cells were starved for 24 hrs in SFM. Immunoblot of pAkt and pMAPK (ERK1/2) from FB-2 treated or not (control) with T3 (10^{-7} M) for 10 and 30 min. The expression of total

AKT and total MAPK was analyzed as a control for gel loading. The side panels represent the means SD of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. *p<0,005 vs control. (B) RNA interference experiments to silence $TR\beta_1$ were performed as described in Materials and methods on FB-2 cells exposed or not to T3 (10^{-7} M) for 20 min. Western Blot analyses showed a specific band corresponding to the phosphorylated Akt (Ser 473) phosphorylated ERK1/2 and $TR\beta_1$. The expression of total Akt, total MAPK and GAPDH were analyzed as controls for gel loading. The side panels represent the means ± SD of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%; *p<0,01 vs control; ° p<0,05 vs T3-treated cells transfected with control siRNA.. (C) Cells were starved for 24 hrs in SFM, pre-incubated with RGD peptide (50nM) for three hours and then treated for 20 minutes with T3 (10^{-7}M). Western Blot analyses showed a specific band corresponding to the phosphorylated ERK1/2. total MAPK was analyzed as a control for gel loading. The side panels represent the means ± SD of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. ** p<0,001vs T3-treated cells; * p<0,005 vs control.

TRβ$_1$ complexes with p85α subunit of PI3K in a ligand independent manner

Recently, some authors *(Cao et al., 2005; Storey et al., 2006, Verga Falzacappa et al., 2009),* in different cell lines have shown an interaction between the subunits p85α of PI3K and TRβ$_1$, similar to that observed for the TR α$_1$ in endothelial cells *(Hiroi et al, 2006).* In our cellular system, we evaluated the interaction between cytoplasmatic TRβ$_1$ and PI3K p85α subunit.

FB-2 cellular extracts immunoprecipitated with anti-TRβ$_1$ antibody showed a constitutive association with the catalytic subunit of PI3K p85α, that was not influenced by rapid treatment with T3 (Fig.3).

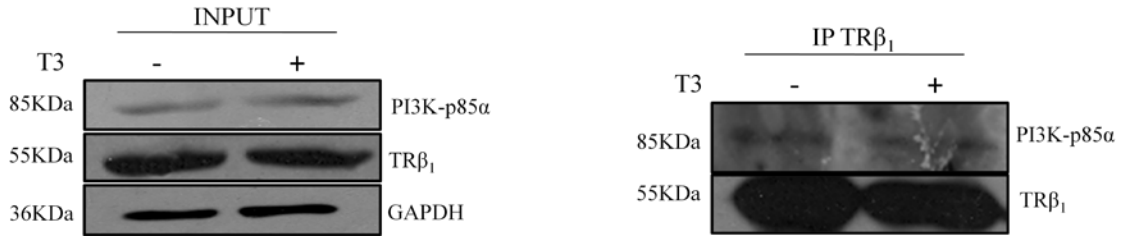


Fig. 3. Coimmunoprecipitation (IP) experiments . Cells were exposed to T3 (10^{-7}M) for 20 min. Immunoprecipitation experiments for TRβ$_1$ were performed on total extracts. Western blot analyses for PI3Kp85a and TRβ$_1$ were performed as described in materials and methods. Whole-cell lysated (INPUT) were used as input controls. All the data shown are representative of at least three independent experiments.

T3 induces TRβ$_1$–associated PI3K activity and promotes the nuclear translocation of activated Akt

To investigate whether T3 is able to affect the PI3K activity, we performed a PI3K assay on TRβ$_1$ pulled down samples. As shown in Fig. 5A, T3 (10^{-7} M) treatment provoked, after 20 minutes, a significant increase in the kinase activity of the TRβ$_1$-associated PI3K. Moreover, to evaluate if the concomitant increase in Akt phosphorylation was accompanied by an up-regulation in the Akt kinase activity, we analyzed the activation of the mean target of Akt activity: glucogen synthetase kinase β that is phosphorylated by Akt on Ser 9 residues. Interestingly, we found that the phosphorylation of GSK3β$_{Ser9}$ was increased by T3 treatment, after 20 minutes. All these data suggested that in FB-2 cells, the rapid T3 treatment is able to induce PI3K activity and its downstream effectors. Besides, as shown in Fig 5 C, the rapid thyroid hormone treatment induces the nuclear translocation of phosphorylated Akt.

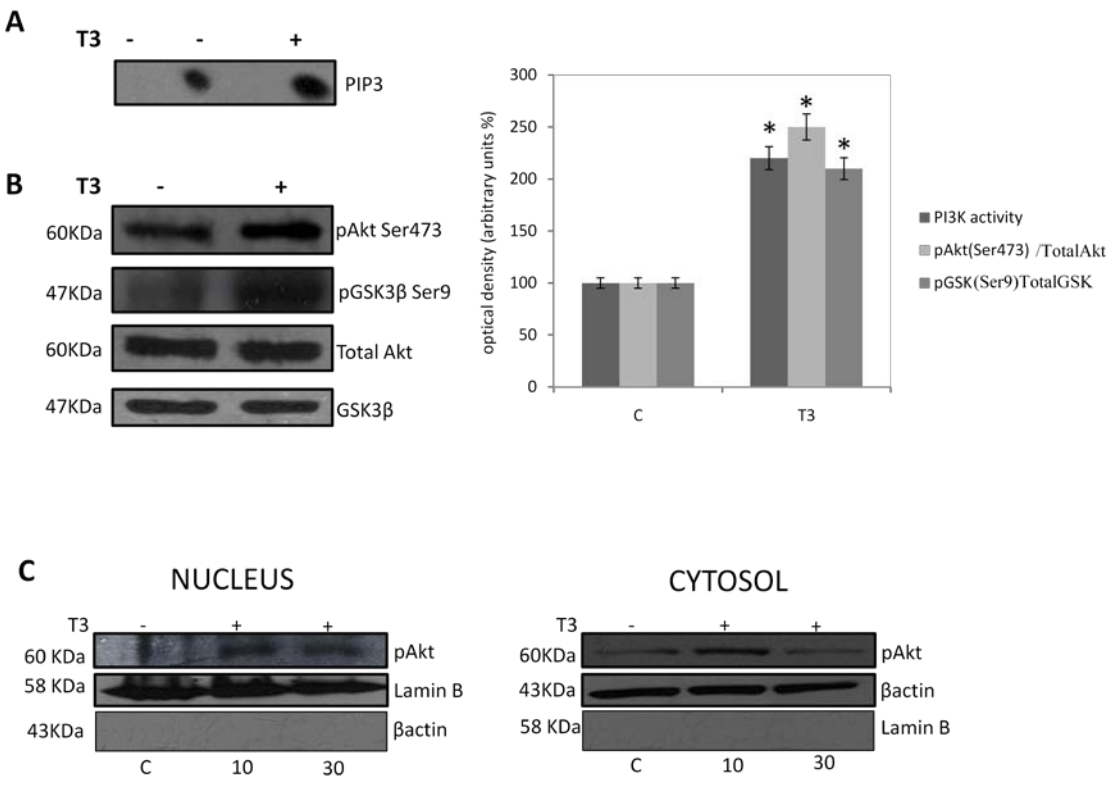


Fig 4. T3 induces TRβ_1–associated PI3K activity and Akt activity**. A**. FB-2 cells were incubated in absence or in presence of T3 100nM for 20 min. Cell lysates were used for PI3K activity. The autoradiograph presented is representative of experiments that were performed at least three times. **B**. FB2 cells were exposed to T3 (10^{-7} M) for 20 min before lysis. Western blot analyses were performed as described in Materials and Methods and specific bands corresponding to the phosphorylated Akt (Ser 473) and phosphorylated GSK3βSer9, were detected. The expression of total Akt and GSK3β were analyzed as controls. The side panels represent the means SD of three separate experiments in which band intensities were evaluated in terms of optical density arbitrary units and expressed as percentages of the control which was assumed to be 100%. * p<0,01vs control. **C** Western Blot analysis on the cytoplasmic and nuclear fractions of protein extracts from FB-2 cells exposed to T3 treatment for 10 and 30 min were performed and a specific band corresponding to the phosphorylated Akt (Ser 473) was detected. The expression of Lamin B (nuclear) and β-actin (cytosol) was analyzed as a control for gel loading and to exclude the contamination of the cytosol with the nuclear components and vice versa.

Activation of PI3-Kinase and MAP-Kinase is involved in thyroid hormone-induced proliferation in FB2 cells

Finally, we investigated the involvement of PI3K and MAPK pathway in the proliferative effect T3 induced in FB-2 cells. To this aim we performed a thymidine incorporation assay using a specific inhibitors of PI3K and MAPK pathway, respectively LY-294002 and PD-98059, in the presence or absence of T3 treatment.

We found that the combined treatment with LY (5µM) or PD (10 µM), significantly reduced the proliferation in cells treated for 48 hours with T3 (10^{-7} M) (Fig. 7), suggesting that in our cellular system, the activation of these pathways is strongly implicated in the proliferative effect induced by thyroid hormone treatment.

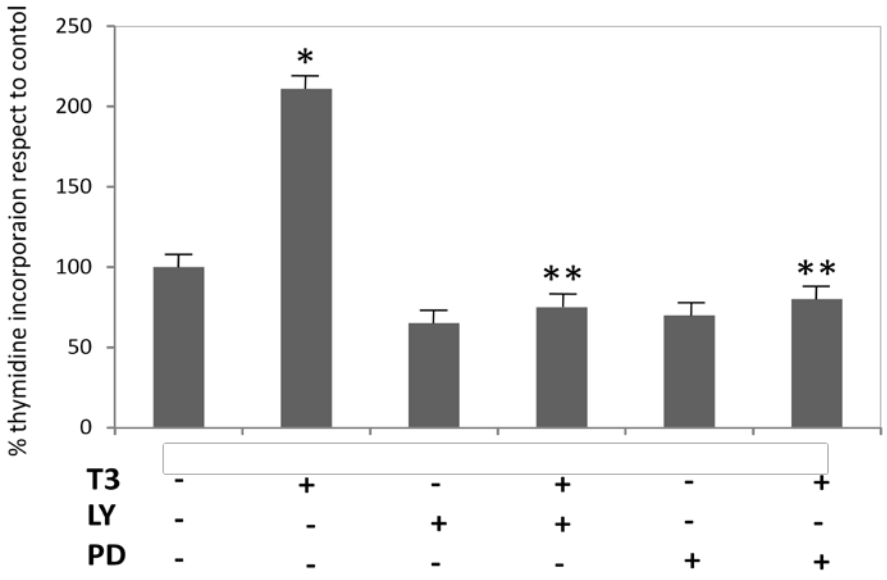


Fig. 7 Activation of PI3-Kinase and MAPK is involved in thyroid hormone-induced proliferation in FB2 cells. FB-2 cells were treated for 48 h with T3 (10^{-7} M), LY-294002 hydrochloride (5μM), PD (10 μM), alone or in combination. After 48 hours, (^{3}H) thymidine incorporation was determined by scintillation counting. The results represent the means ± SD of three independent experiments, each performed with triplicate samples and expressed as percentage of growth vs control which was assumed to be 100%. * p< 0,05 treated vs control;. ** p< 0,05 T3 + Ly or PD treated vs T3 treated.

DISCUSSION

Some of the non-genomic actions of TH, involve TRs located outside the nucleus, since it is known that 10% of TRs are cytoplasmatic in absence of T3 (*Baumann et al., 2001*), whereas others utilize other proteins that can bind TH, such as integrin αVβ3 (*Bergh et al., 2005*). Non-genomic mechanisms of TRs appear to be relevant to motility of endothelial cells and to proliferation of several tumor cells; indeed, many authors reported that TH causes in vitro proliferation of a variety of cancer cells, including thyroid cancer cells (*H.Y.Tang et. al 2004, M.Cristofanilli et. al 2005 , F.B.Davis et. al 2006*; *G.B.Hernandez et. al 1999, F.B.Davis et. al 2006, H.Y.Tang et. al 2004*; *M.L.Hsieh and H.H.Juang 2005*; *P. Poplawski et al 2008).*

In the present study we investigated the non-genomic signaling effects induced by thyroid hormone T3 on a novel thyroid papillary human cancer cell line, designated FB-2. Our data demonstrated that T3 short exposure is able to activate MAPK/ERK1/2 signaling as well as PI3K/Akt pathway.

Cellular processes, such as proliferation and survival, induced by different hormones and growth factors are dependent on the activation of PI3K. PI3K is a kinase that consists of a catalytic subunit of about 110KDa (p110) and a tightly associated regulatory subunit (p85α, p85β, or p55y). The subunit regulates the association of PI3K with membrane-associated signaling complexes. Upon activation by membrane receptors, PI3K phosphorylates phosphatidyl-inositol-4,5 biphosphate (PIP2) to form phosphatidyl-inositol-3,4,5-triphosphate (PIP3). Through phosphatidylinositol-dependent kinases, the downstream effectors of PI3K, the serine/threonine Kinase Akt, is phosphorylated and activated to further phoshorylate downstream protein substrate *(Ner el al., 2002; Sheperd et al., 1997; Wymann and Marone, 2005),* leading to various

signaling cascades that affect cellular functions. The activity of PI3K is negatively regulated by PTEN, a protein phosphatase that dephosphorylates PIP3 to form PIP2 *(Eng, 2002).*

The activation of PI3K pathway can occur within minutes or hours, and is related to non-genomic action of specific activators. Recently, it has been observed that liganded or unliganded TRs bind to the p85α subunit of PI3K and activate the PI3K signaling pathway, including the phosphorilation of Akt (serine 473), a mammalian target of rapamycin (mTOR) and its substrate $p70^{S6K}$ *(Hiroi et al., 2006; Verga Falzacappa et al., 2009).* Refetoff et al. showed, in human fibroblast, that T3-treatment induced a rapid mTOR activation, with detectable phosphorylation as early as 10 minutes and not sensitive to cycloheximide treatment, indicating that this effect of thyroid hormones uses preexisting proteins. It is not clear why the mechanism of activation of PI3K signaling by TR differs in the cytoplasm and the plasma membrane, but may be due to different post-translational modifications of $TR\beta_1$ and p85α subunit in the cytoplasm versus the plasma membrane, different receptor/enzyme complex formation, or cell-specific effects *(Moeller et al., 2006; Oetting, 2007).*

Recent studies of human cancer specimens by several groups, showed Akt over-expression and over-activation in primary thyroid cancers (*Ringel et al., 2001; Miyakawa et al., 2003; Motti et el., 2005*). In particular, Furuya et al., have reported that in a mouse model of follicular thyroid carcinoma ($TR\beta^{PV/PV}$), harbors a knockin mutant $TR\beta_1$ gene, the physical interaction of PV with p85α subunit results in a constitutive activation of the PI3K signaling that leads to an increase of thyroid tumor growth and enhanced cell motility. Moreover, they showed that the inhibition of the PI3K activity by LY decreased cell proliferation, suggesting that LY treatment, in combination with

other specific inhibitors of downstream effectors of PKB, could provide additional therapeutic effectiveness in treating thyroid cancer.

Tumors of follicular thyroid cells are highly heterogeneous in terms of histology and response to treatment. Malignant thyroid tumors include (a) well differentiated carcinomas, which comprise papillary (PTC) and follicular (FTC) carcinomas; (b) poorly differentiated carcinomas; and (c) undifferentiated carcinomas *(Basolo et al, 2002).* Well-differentiated PTC is the most frequent type of thyroid cancer and it accounts for the vast majority of thyroid carcinomas associated with previous exposure to ionizing radiation. The prognosis of papillary thyroid carcinoma is generally favorable. However, a number of patients develops recurrences (local or nodal) and distant metastases, or dies *(Mazzaferri et al, 1999).* The molecular mechanisms underlying the initiation and progression of thyroid carcinoma are not full understood, but it is generally believed that deregulation of cell growth and cell death is involved.

In this study we demonstrated that in FB-2 cells, $TR\beta_1$ receptor was clearly detectable both at the cytoplasmatic and at nuclear level, confirming its typical localization and its expression was not affected by the rapid thyroid hormone treatment. It has been recently reported that thyroid hormone receptor β_1 is involved in the proliferative effect induced by thyroid hormone T3 in human insulinoma cell line *(Verga Falzacappa et al., 2009).*

To explore the involvement of thyroid hormone receptor β_1 in the proliferative effect induced by T3 in FB-2 cells, we performed proliferative assay using a specific "knockdown" of the gene encoding $TR\beta_1$, through experiments of RNA interference. Interestingly, our data showed that the T3 effect on proliferation was completely abolished in the $TR\beta_1$ silenced cells, demonstrating the involvement of this receptor for the T3 induced proliferation in FB-2 cells.

The non-genomic mechanisms of thyroid hormone action include activation of the ERK1/2 signal transduction pathway and PI3K signaling. Since in our study we showed that T3 is able to induce a rapid phosphorylation of Akt and ERK1/2, we explored the involvement of thyroid hormone receptor β_1 in the activation of these pathways. Our data demonstrated that the "knockdown" of the gene encoding TRβ_1, results in a abolishment of AKT activation T3-induced, while the phosphorylation of MAPK was not affected. It's well known that the proliferative effect exerts in vitro by thyroid hormone in some cancer cells, is membrane-initiated at a hormone receptor site on integrin αVβ3 through which thyroid hormone non-genomically actives the MAPK signal transduction cascade *(Oetting, 2007; Davis et al., 2007).* To verify the role of integrin receptor in hormone induced activation of MAPK in FB2 cells, we used the well known RGD peptide that displace thyroid hormone from integrin, blocking MAPK-activation. Our data evidenced that in the presence of RGD peptide an inhibition of MAPK activation T3-induced was observed.

It has been evidenced the action of the thyroid hormone T3 on the PI3K/Akt pathway, showing that T3 can stimulate the phosphatidylinositol 3-kinase at the plasma membrane *(Cao et al., 2005; Furuya et al., 2006; Verga Falzacappa et al., 2006, Lin et al., 2009).* This activation involves the binding of the TRβ_1 and the subunit p85α of the PI3K: the said interaction has been proved to be both nuclear and extranuclear *(Furuya et al., 2006).* The activation of the PI3K leads to events that include the triggering of the AKT Kinase and its downstream mTOR. These evidences have suggested that the action of TRβ_1 on the PI3K pathway might drive the increment of cell proliferation and the suppression of apoptosis.

Our findings showed that in FB2 cells, thyroid hormone receptor β_1 is able to form a complex with the subunit p85α of PI3K in a ligand-independent manner;

however, nanomolar concentrations of T3, enhances the $TR\beta_1$-associated PI3K activity. In addition, T3-induced kinase activation can trigger a cascade of events that are PI3K-dependent, including a rapid phosphorylation of Akt at Ser 473 and of GSK3β at Ser 9, one of the main Akt substrates.

In the biological counterpart of the present study, we evidenced the involvement of PI3K pathway and MAPK signaling in the growth effect induced by T3 in FB-2 cells, since in the presence of LY or PD the proliferation of FB-2 cells induced by T3 treatment was reversed. All these findings demonstrate how the short exposure to T3 induces tumor cell proliferation concomitantly with anti-apoptotic actions.

If this proliferative action of thyroid hormone in supra-physiological concentrations is reproduced in the intact organism, it has several implications that are specific for thyroid cancer. First, the coexistence of thyroid cancer (TC) and hyperthyroidism in patients is well described, with a reported incidence highly variable, ranging between 0,2% and 21,0%; moreover, is reported that in toxic thyroid carcinoma, the papillary hystotype is predominant (*Gabriele et al, 2003;Vaiana et al., 1999*; *Gulcelik et al., 2006;).* Such a wide range of incidence may be due to different study protocols, diagnosis and follow-up, but the most important factor may be variable iodine intake of the patients living in different area of iodine supply (*Cappelli et al., 2006; Gulcelik et al., 2006; Pazaitou-Panayiotou et al., 2008; Giles et al., 2008*). However, the nature of "hyperthyroidism-thyroid cancer" relationship is yet controversial. Experimental data and clinical reports suggest that a patho-physiological role may be exerted by TSH that not only stimulates normal thyroid cell growth and function but may promote changes favorable to subsequent tumor development before that the toxic status suppressed its concentration. Second, it is not clear whether T3 or T4 influence the growth of normal thyroid or thyroid tumors (*Lin et al., 2003*);

therefore, the conventional use of exogenous thyroid hormone to suppress endogenous TSH in patients with multinodular goiter or in the thyroid cancer patient who has undergone thyroidectomy and radioablation, may be carefully considered, in view of the growth-promotion action of T3 on tumor thyroid cells.

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1) Bonofiglio D, Catalano S, **Perri A**, Baldini MP, Marsico S, Tagarelli A, Conforti D, Guido R, Andò S. *Beneficial effects of iodized salt prophylaxis on thyroid volume in an iodine deficient area of Southern Italy.* Clin Endocrinol (Oxf). 2008 Sep 23.

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Clin Endocrinol (Oxf). Jul 2007;67(1):34-4

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Comunications in National and International Conferences during the Program

1) Workshop “L’Ipotiroidismo Congenito in Italia, Istituto Superiore di Sanità” (Roma, 4 Luglio 2007)

2) Workshop “La iodoprofilassi in Italia, Istituto Superiore di Sanità” (Roma, 5 Luglio 2007)

3) VI Corso teorico-pratico di citologia e citopatologia della tiroide (ROMA, UNIVERSITA' CATTOLICA DEL SACRO CUORE - 28/29 NOVEMBRE 2007)

4) Primo Congresso multidisciplinare sull’acne - 19 gennaio 2008 – Pisa

5) XXIX National Congress Italian Society of Pathology (University of Calabria Rende (CS) Italy, September 10-12 2008)

6) Corso SIAMS Calabria – “Impotenza che fortuna!” (Cosenza, 9 Maggio, 2009): *Relazione dal titolo “Terapia del “Late-onset hypogonadism”*

7) 33° Congresso Nazionale Società Italiana di Endocrinologia (Sorrento, 27-30 maggio 2009): *POSTER dal titolo “Rapid c-Src mediated effects induced by triiodothyronine on proliferative and survival signaling in FB-2 papillary thyroid carcinoma cells”*

8) Workshop “L’ipotiroidismo congenito”, Istituto Superiore di Sanità” (Roma, 4 Luglio 2009)

9) Workshop “La iodoprofilassi in Italia”, Istituto Superiore di Sanità” (Roma, 5 Luglio 2009)

10) Corso ECM “La iodoprofilassi per la prevenzione del gozzo endemico e delle patologie ad esso correlato, Università della Calabria (Rende, 28 novembre 2009). Relazione del titolo “Linee guida sulla gestione del paziente con patologia tiroidea: ruolo del Medico di Medicina Generale, del Pediatra e dello Specialista”.

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ORIGINAL ARTICLE

Genetic analysis of the *PAX8* gene in children with congenital hypothyroidism and dysgenetic or eutopic thyroid glands: identification of a novel sequence variant

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Summary

Objective To analyse the coding region of *PAX8* in individuals with congenital (CH) or post neonatal hypothyroidism due to dysgenetic (TD) or eutopic thyroid glands.

Design and patients Forty-three children with CH and TD (13 agenesis, 23 ectopia, and seven hypoplasia), one subject with post neonatal onset of hypothyroidism and thyroid ectopia, 15 children with CH and eutopic thyroid glands and six euthyroid adults with thyroid hemiagenesis were enrolled as cases, along with 120 healthy individuals as controls.

Measurements Exons 2–8 of the *PAX8* were directly sequenced. HeLa and HEK293 cells were transfected with *PAX8* wild-type (*PAX8*-WT), mutant *PAX8*, p300, thyroid transcription factor 1 (*TTF-1*) and thyroglobulin promoter pGL3 (*TG* prom-pGL3). Synergism of *TTF-1* with *PAX8*-WT *vs.* mutant and activity of *PAX8*-WT *vs.* mutant in accompaniment with p300 on *TG* prom-pGL3 were also assessed. The luminescence produced by *PAX8*-WT and mutant *PAX8* was measured.

Results Among patients and controls only a 15-year-old girl with thyroid ectopia showed a heterozygous transition of cytosine to thymine at position 674 in exon 6, which changed a conserved threonine at position 225 to methionine (*PAX8*-T225M). Her father and sister harboured *PAX8*-T225M without abnormal thyroid phenotypes. *PAX8*-T225M and *PAX8*-WT similarly increased luciferase activity and had a similar synergistic effect with *TTF-1*. At 500 ng p300, however, *PAX8*-T225M could not significantly increase *TG* promoter activity when compared to *PAX8*-T225M alone, while *PAX8*-WT +500 ng p300 induction was significantly higher than *PAX8*-WT alone ($P < 0{\cdot}001$). Cotransfection of *TTF-1* together with *PAX8*-T225M resulted in rescuing of the lack of synergism with p300.

Conclusions *PAX8* mutations in congenital hypothyroidism due to dysgenetic or orthotopic thyroid glands are rare. *PAX8-T225M* is probably a rare variant.

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Introduction

Primary congenital hypothyroidism (CH) is the most common congenital endocrine disorder, occurring in 1 in 3000–4000 live births.[1] In iodine replete areas, thyroid developmental defects (75–80%) and inborn errors of thyroid hormone biosynthesis (10–15%) are the most frequent, while transient (5–10%) and central (hypothalamic–pituitary) hypothyroidism (approx. 5%) are the least frequent causes of CH.[2,3] Thyroid dysgenesis (TD) presents as agenesis (athyreosis), hypoplasia, ectopia or hemiagenesis of the thyroid gland.[4,5] The pathogenesis of TD is largely unknown.[6] While most cases are sporadic, up to 2% of patients with TD have a positive family history of this condition.[7] Although there is discordance of TD among monozygotic twins[8], mild thyroid abnormalities in first degree relatives of patients with TD,[9] the association of parental consanguinity and TD occurrence in a population with high blood-related marriages[10] and a recent report on the mouse model suggestive of a multigenic origin of TD[11] suggest that the mechanisms underlying thyroid dysgenesis are complex and heterogeneous. The current data suggest that there is a spectrum ranging from monogenic to multifactorial genetic aetiologies, and environmental and epigenetic modifiers are likely to be contributing factors.[12,13]

PAX8, a transcription factor of the mammalian Pax protein family, is known to recognize DNA via a highly conserved paired domain.[14–16] It is detected in the developing thyroid in early embryonic days at the time of specification and its expression is maintained in thyroid follicular cells during all stages of development.[17,18] The *PAX8* gene maps to human chromosome 2q12–2q14 and consists of 11 exons.[4,19] Initial screening of 145 patients with known TD revealed *de novo* monoallelic mutations of *PAX8* in two sporadic cases and in a family with three CH-affected members.[20] Subsequently, other mutations

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have been discovered.[21–25] In the familial cases transmission was autosomal dominant.[18] However, in one family there was profound discrepancy between two related individuals bearing the same heterozygous mutation denoting that the phenotype is highly variable even with the same mutation.[22] Recently, the novel mutation (S48F) located in the *PAX8* paired domain has been shown to have a specific defect in transactivation[26] due to a loss of co-operation with the general co-activator p300.

In this study we performed mutational analyses of the *PAX8* gene in CH-affected individuals (and in one with post neonatal hypothyroidism) with dysgenetic and orthotopic thyroid glands.

Subjects, materials and methods

Subjects

The study population comprised 43 children with CH and thyroid dysgenesis, either agenesis, ectopia, or hypoplasia, one subject with post neonatal onset of hypothyroidism and thyroid ectopia (group 1), six young adults with thyroid hemiagenesis without clinical or biochemical hypothyroidism (group 2), 15 children with CH and eutopic thyroid gland of normal size (group 3) and 120 consecutively selected healthy volunteers with no thyroid abnormality (group 4). Except for one subject in group 1, CH was diagnosed in all group 1 and group 3 children during national newborn screening for CH using the primary thyrotropin (TSH) screening test. Levothyroxine (L-T4) replacement treatment, 10–12 μg/kg/day, was started within the first month of life and L-T4 dosage was adjusted during infancy and childhood according to serum TSH and thyroxine (T4) in follow up sessions (2). Determination of CH type (permanent *vs.* transient), after 30 days of L-T4 therapy discontinuation, and of permanent CH aetiologies (dysgenesis *vs.* eutopic thyroid glands) by thyroid scintigraphy using technetium-99 m pertechnetate (^{99m}TC) were performed at ≥ 3 years of age in CH-affected individuals.

Group 1

Forty-three subjects in this group were born at term (≥ 37 weeks' gestation) after an uneventful pregnancy. Thyroid ^{99m}Tc scan was performed during the 6th year of age after 30 days of discontinuation of L-T4 replacement therapy, and subsequent elevation of serum TSH confirmed the permanent nature of CH. Thyroid scintigraphy revealed absent uptake (agenesis confirmed by subsequent ultrasonography) in 13, ectopia in 23, and hypoplasia in seven individuals. On physical examination no craniofacial, truncal or limb anomalies were found and no structural visceral, in particular cardiac and renal, anomalies were observed upon echocardiography and abdominal ultrasonography.

The last patient in this group, a female subject, had been diagnosed with hypothyroidism at 15 years of age during the evaluation of primary amenorrhoea. At the time of diagnosis she had a serum TSH of 71 (normal: 0·3–4) mU/l, free T4 (FT4) of 7·7 (normal: 10·3–24·5) pmol/l, and free triiodothyronine (FT3) of 4·6 (3·1–7·5) pmol/l. TPOAb and TgAb were negative. Cervical ultrasonography showed a 10-×-11-mm round mass at the base of the tongue and ^{99m}Tc scanning confirmed the presence of ectopic thyroid tissue. Her past medical record showed that she was born at term after an uncomplicated pregnancy and had normal TSH on neonatal screening testing. It remains unclear when biochemical and clinical hypothyroidism first appeared. Nevertheless, no signs of mental retardation were present at the time of CH diagnosis. At 17 years of age her height was 157 cm (25th percentile for the age- and sex-matched Italian population) and her weight was 39·5 kg.

Group 2

Six adult subjects (mean age of 20·5 years) with clinical and biochemical euthyroidism and thyroid hemiagenesis, detected by ultrasonography, were included in the study. All subjects showed hemiagenesis of the left thyroid lobe.

Group 3

This group comprised 14 children born at ≥ 37 weeks' gestation after uneventful pregnancies and a premature female offspring of a twin delivery. All had abnormally high TSH at newborn screening and serum TSH and T4 confirmed CH diagnosis during neonatal and infancy periods. Cervical ultrasound showed a bi-lobed, properly located and normal-sized thyroid gland in all of the subjects during the neonatal period.

In six children aged ≥ 3 years old, permanent CH was diagnosed by abnormal serum TSH and FT4 after 30 days of L-T4 discontinuation, and orthotopic thyroid glands were also confirmed by ^{99m}Tc thyroid scans. In the remaining eight children L-T4 therapy was not stopped because they were < 3 years of age.

The only premature subject, product of a provisionally monozygotic twin delivery according to phenotype similarities, was born at 28 weeks' gestation after an uneventful pregnancy. CH was diagnosed via a neonatal screening programme and subsequent ultrasonography revealed in-place thyroid gland, which was also confirmed by thyroid ^{99m}Tc scintigraphy during the neonatal period. L-T4 replacement therapy, 10 μg/kg/day, was started at 19 days of life. Her twin sister had normal TSH at CH screening.

The study was approved by the local ethical committee of our university and informed consent was obtained from all the parents of the children.

Thyroid ultrasound

Ultrasound evaluation was performed by the same examiner (M.T.) using a linear transducer (7·5 MHz) attached to a real time instrument (AU 590 Asynchronous Apparatus, Esaote Biomedica, Milan, Italy).

Laboratory evaluation of thyroid function

TSH was assessed by sensitive-TSH IRMA (Delfia, Wallac, Finland). FT4 and FT3 were measured in the laboratory of our department by radioimmunoassay (FT4 RIA, FT3 RIA, Lysophase; Technogenetics, SpA, Milan, Italy). TPOAb and TgAb were measured by passive agglutination (SERODIA-AMC and SERODIA-ATG, Fujirebio, Tokyo, Japan).

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Direct sequencing of the PAX8 *gene*

After obtaining written consent from the children's parents and from the adult subjects for participation in the study, blood was collected and genomic DNA was extracted from 200-μl peripheral blood leucocytes, using the QIAamp® DNA Blood Mini Kit (Qiagen S.p.A., Milano, Italy). Specific primers were used to sequence human *PAX8* exons and also flanking introns. Exons 2–8 of the *PAX8* gene[20] were amplified by PCR in 50 μl using 300 ng of genomic DNA, 1 pmol/μl of the primers supplemented by 20 mM Tris HCl (pH 8·4), 50 mM KCl, 1·5 mM $MgCl_2$, 10% dimethyl sulfoxide (DMSO), 200 μM dNTPs, and 0·5 U of recombinant Taq DNA polymerase (Invitrogen S.r.l, Milan, Italy). PCR conditions were as follows: 2 min at 95 °C, 1 min at 94 °C, 1 min at annealing temperature, 1 min at 72 °C for 30 cycles and 5 min at 72 °C.

PCR products obtained were purified with Mini Elute™ PCR Purification Kit (Qiagen S.p.A.), automatically sequenced using dye terminator cycle sequencing (DTCS) with a Quick Start Kit (Beckman Coulter S.p.A., Milan, Italy), and analysed on a DNA sequencer machine (Beckman Coulter, model CEQ™ 2000XL DNA Analysis System, Fullerton, CA). Sequences were compared with the human full-length cDNA *PAX8* (*PAX8* isoform) sequence previously reported.[15] The intronic sequences close to the splice sites were also analysed.

Direct sequencing of the TTF-1 *gene*

The entire coding sequence of *TTF-1* was also assessed in the extracted DNA of the subject with a monoallelic mutation in *PAX8*. Exons were amplified by PCR in 50 μl using 300 ng of genomic DNA, 1 pmol/μl of primers 5′GCTTCGCCTTCCCCCTCTCCCTTTT3′, 5′GACGGCTCTCGCCGCACCTCCTGAG3′, 5′GCGCGGAAAACAGGGGTGGC3′, 5′CCAGAACCACCGCTACAAAATGAAG3′, and 5′GCTGCGCCGCCTTGTCCTTG3′ (exons 2 and 3), and Master Mix (Promega Corporation, Madison, WI). The PCR conditions were as follows: 5 min at 94 °C, 0·7 min at 94 °C, 1 min at 60 °C, 1·5 min at 68 °C for 35 cycles and 10 min at 72 °C. In addition to the primers used for PCR, the following primers were also used for the sequencing reactions of PCR products: 5′CGCCTACCACATGACGGCGGCGGGG3′, 5′CTCATGTTGCCCAGGTTGCCGTTGC3′, and 5′AGGGCCGGCCCGGCGTCCTCTCACC3′. Purification of PCR products and direct sequencing followed the same protocol as described above for *PAX8*.

Functional analysis of the mutant

Expression vector and reporter gene constructs. PAX8 cDNA was obtained by reverse transcriptase PCR of mRNA extracted from human thyroid tissues using 5′ATATGGTACCATGCCGCACAACTCCATC3′ and 5′ATATTCTAGACTACAGATGGTCAAAGGC3′ primers. The PCR product was cloned in the pcDNA3 expression vector, using *Kpn*I and *Xba*I restriction sites introduced in the primers to obtain *PAX8*-WT-pcDNA3.

The pGL3 luciferase reporter vector contains the cDNA encoding luciferase cloned from the North American firefly and a vector backbone that has been designed to provide enhanced reporter gene expression. Human thyroglobulin prom-pGL3 (*TG* prom-pGL3), which contains the *TG* promoter and the luciferase cDNA encoding the modified firefly luciferase (i.e. the reporter), *TTF-1*-pcDNA3,[27] and pCMV-HA-p300 expression vectors have previously been described.[26]

The *PAX8*-T225M-pcDNA3 mutation was created by site-directed mutagenesis with an overlap extension strategy, using 5′AAGCACCTTCGCATGGATGCCTTCAGCCAG3′ and 5′CTGGCTGAAGGCATCCATGCGAAGGTGCTT3′ primers containing the mutant base and using 5′ATATTCTAGACTACAGATGGTCAAAGGC3′ and 5′ATATGGTACCATGCCTCACAACTCCATC3′ primers for the recombinant PCR.[26]

Cell cultures and in vitro *functional assay*

Activity of PAX8-*T225M-pcDNA3 and* TTF-1-*pcDNA3 on* TG *promoter.* HeLa cells were grown in DMEM (Invitrogen, Inc., Milan, Italy) supplemented with 2 mM L-glutamine, 25 mmol/l D-glucose, 50 U/ml Penicillin, 50 μg/ml streptomycin, and 10% fetal bovine serum under humidified 5% CO_2/95% air at 37 °C. HeLa cells (3×10^5) were plated in 35-mm-diameter culture dishes for 24 h before transfection. Transfections were carried out in duplicate with FuGENE 6 Transfection Reagent (Roche Diagnostic Corporation, Indianapolis, IN) following the manufacturer's instructions. HeLa cells were transfected with *PAX8*-WT-pcDNA3 (1 μg), *PAX8*-T225M-pcDNA3 (1 μg), *TTF-1*-pcDNA3 (0·5 μg), together with *TG* prom-pGL3 (1 μg). After 48 h the catalytic action of luciferase in oxidation of luciferin and, subsequently, luminescence production in cell extracts was assayed using the Lumino (Stratec Electronic; GMBH, Birkenfeld, Germany) luminometer. Light intensity was quantified using a preproduced standard curve and was reported in relative light units (RLUs).

Activity of PAX8-*T225M-pcDNA3 and pCMV-HA-p300 on* TG *promoter (*TG *prom-pGL3).* HEK293 cells (2×10^5 cells per well) were grown in 12-well plates to 70–80% confluence and transfected with *PAX8*-WT-pcDNA3 (15 ng), or *PAX8*-T225M-pcDNA3 (15 ng) together with 0·5 μg *TG* prom-pGL3, and with pCMV-HA-p300. The same experiments were also performed in the presence of *TTF-1*-pcDNA3 (0·8 μg).

pRL-TK (Promega Corporation) containing *Renilla* luciferase gene was also transfected as an internal control vector. Various doses of effector plasmids/empty pcDNA3 vector using FuGENE 6 reagent (Roche Diagnostic Corporation) were used. Cells were harvested 48 h later and analysed sequentially for firefly and *Renilla* luciferase activities by the Dual-Luciferase Reporter Assay System (Promega Corporation).

Results

Direct sequencing of PAX8

Direct sequencing of exons 2–8 of *PAX8* revealed the presence of a heterozygous transition of cytosine to thymine (C→T) at position 674 relative to the translation initiation codon of the human *PAX8* coding sequence, which changed ACG code to ATG and a conserved

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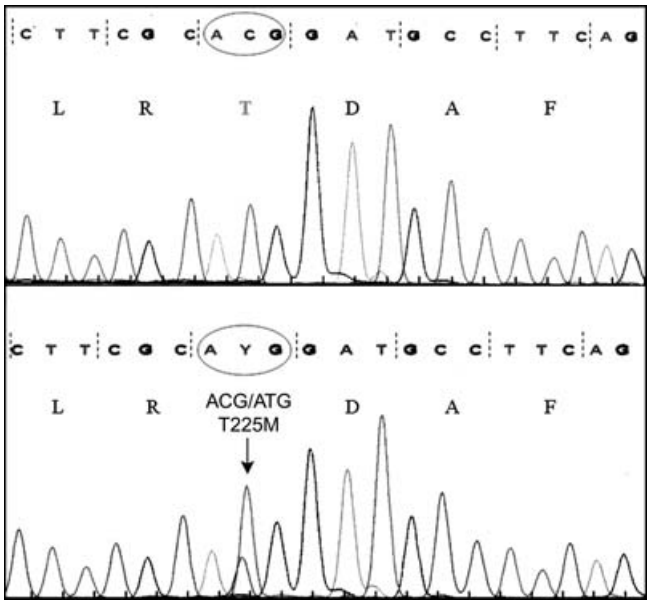

Fig. 1 Sequencing electropherogram of part of exon 6 showing a heterozygous C→T transition at nucleotide 674 of the *PAX8* coding sequence. This mutation replaces a conserved threonine at position 225 to methionine (T225M).

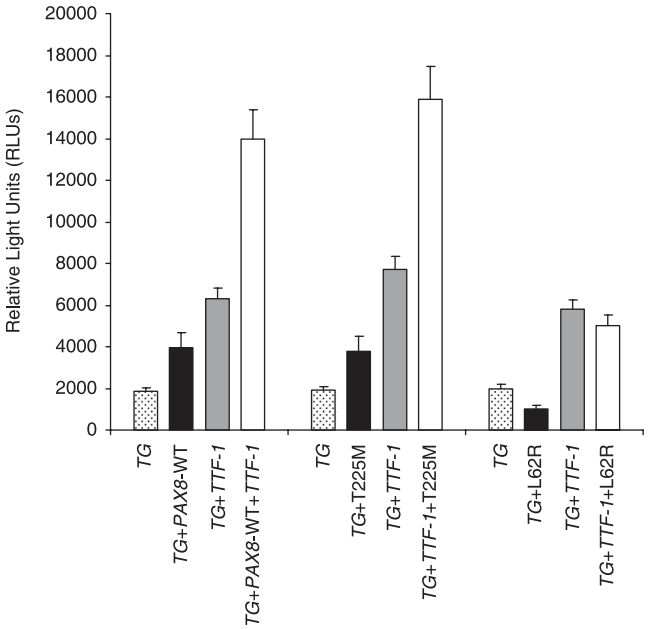

Fig. 2 HeLa cells were cotransfectd with a constant amount of either *PAX8*-WT or *PAX8*-T225M expression vectors along with *TTF-1* expression construct. For each transfection, the total amount of DNA was kept constant by adding empty vector. Data are reported as relative light units (RLUs). *PAX8*-WT and *PAX8*-T225M constructs show similar synergistic activity when cotransfected with *TTF-1*-pcDNA3. The well-known loss-of-function mutation *PAX8-L62R* (20) induced a remarkable decrease in luciferase activity when cotransfected with the *TG* promoter. Similarly, cotransfection of *PAX8*-L62R and *TTF-1* was not accompanied by a synergistic effect.

threonine (T) at position 225 to methionine (M), i.e. *PAX8*-T225M (Fig. 1). The mutation was detected in exon 6, outside the paired domain[25,28,29] in a CH-affected female subject with thyroid ectopia. Further evaluation showed that *PAX8*-T225M was also present in one allele of her father and sister, while it was of wild type in the mother. The father and the sister were clinically and biochemically euthyroid, with a normal thyroid gland in the proper position in the neck. *PAX8*-T225M was not observed, either in other thyroid dysgenetic and eutopic CH-affected individuals or in 120 healthy control subjects of the study.

Direct sequencing of the *PAX8* gene also showed a substitution of T→C at position 994 in exon 8 of three (two agenetic and one ectopic) CH cases, resulting in the change of TTT to CTT codon and the subsequent change of phenylalanine (F) at the position of 329 to leucine (L), i.e. *PAX8*-F329L. No further functional analyses were performed for this nucleotide sequence change as this is a known polymorphism.[30] None of the other patients in any of the groups harboured *PAX8* gene mutations.

Direct sequencing of TTF-1

Direct sequencing of *TTF-1* in the index case carrying *PAX8*-T225M was of wild type.

Capacity of activation TG *prom-pGL3 by* PAX8-*WT-pcDNA3 and* PAX8-*T225M*

To evaluate the functional significance of the *PAX8*-T225M mutation we tested the ability of the mutant protein to activate a cotransfected reporter gene under the control of the human *TG* promoter. Cotransfection of *PAX8*-WT expression vector into HeLa cells produced up to 2-fold stimulation of luciferase activity compared to cotransfection with an empty vector. Similarly, co-expression of the *PAX8*-T225M mutant in HeLa cells produced a comparable stimulation of luciferase activity (Fig. 2). In comparison to the activity of *TTF-1* alone there was approximately a 2·3-fold increase in the activity of luciferase when *PAX8*-WT and *TTF-1* were cotransfected. This synergistic effect was also of similar intensity when *TTF-1* was cotransfected with the *PAX8*-T225M (Fig. 2). Therefore, it seems that *PAX8*-T225M has not lost its ability to bind a specific DNA sequence and activate, in synergy with TTF-1, transcription of the *TG* promoter.

Cotransfection of HeLa cells showed that *PAX8*-T225M has no dominant-negative effect on the activity of *PAX8*-WT (data not shown).

There is evidence that the PAX8 protein interacts with a number of other transcription factors, forming complexes on regulatory regions of target genes. The general transcriptional coactivator p300 has previously been shown to be essential in mediating *PAX-8* activation on the *TG* promoter, in *PAX8* activity on the rat thyroperoxidase (TPO) promoter and in mediating the functional synergism between *PAX8* and *TTF-1* in thyroid-specific gene expression.[26,31] In order to assess whether *PAX8*-T225M was able to efficiently recruit p300 and assemble the transcriptional coactivation complex[26] we employed HEK293 cells. These cells are deficient in endogenous p300 because of expression of the adenovirus E1A protein,[32] which sequestrates p300 in the cytosol. p300 was cotransfected with *PAX8*-WT and with

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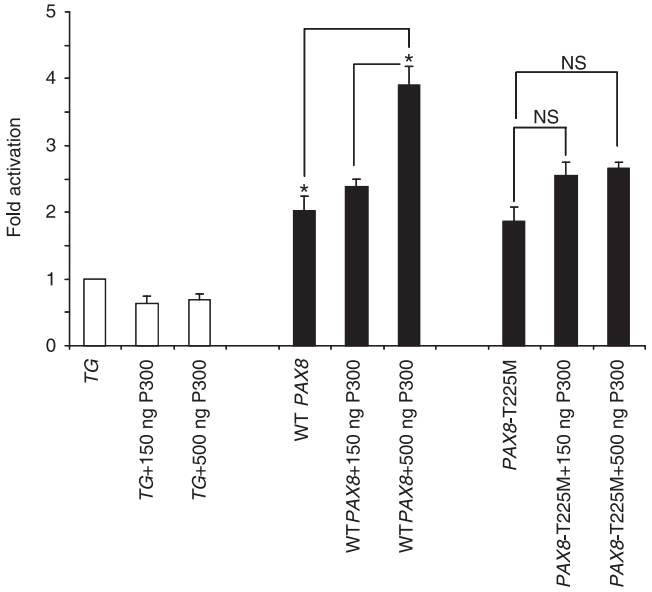

Fig. 3 HEK293 cells were cotransfectd with a constant amount of either *PAX8*-WT or *PAX8*-T225M expression vectors along with increasing amounts of p300 expression construct and *TG* prom-pGL3. For each transfection, the total amount of DNA was kept constant by adding empty vector. Firefly luciferase activities were normalized to the *Renilla* luciferase activity derived from cotransfected pRL-Tk internal control vector and represented relative to the activity obtained by cotransfection of *TG* prom-pGL3. Results are the mean ± SD of three independent experiments performed in triplicate (*indicates $P < 0{\cdot}001$).

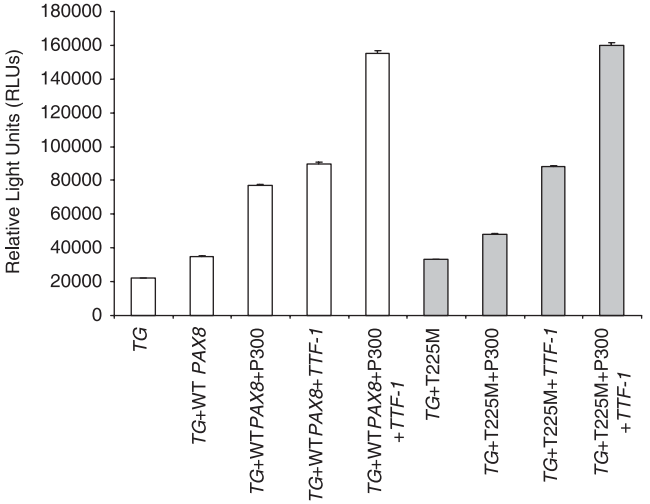

Fig. 4 HEK293 cells were cotransfected with a constant amount of either *PAX8*-WT or *PAX8*-T225M expression vectors along with p300 expression construct, *TTF-1*-pcDNA3 and *TG* prom-pGL3. For each transfection, the total amount of DNA was kept constant by adding empty vector. Firefly luciferase activities were normalized to the *Renilla* luciferase activity derived from cotransfected pRL-Tk internal control vector and represented relative to the activity obtained by cotransfection of *TG* prom-pGL3. Results are the mean ± SD of three independent experiments performed in triplicate.

PAX8-T225M together with an internal control (pRL-TK *Renilla*) and the *TG* promoter (Firefly). The firefly to *Renilla* luminescence activity ratios were calculated and compared between groups.

There was a dose-dependent increase in *TG* promoter activity either cotransfecting *PAX8*-WT plus different concentrations of p300 or *PAX8*-T225M plus the same concentrations of p300 (Fig. 3). However, this pattern was different in wild-type and mutant groups. Indeed, *PAX8*-WT plus 500 ng p300 revealed significantly increased activity in comparison to *PAX8*-WT alone ($P < 0{\cdot}001$). *PAX8*-T225M did not show significantly increased *TG* promoter activity with 150 and 500 ng of p300 with respect to *PAX8*-T225M alone. Cotransfection of TTF-1 resulted in rescuing of the synergistic effect of p300 on *PAX8*-T225M-mediated transactivation (Fig. 4).

Discussion

The *PAX8* gene is required for both morphogenesis of the thyroid gland and maintenance of the thyrocyte cell type and is essential for the thyrocyte-specific promoter activation of the *TPO*, *TG* and sodium iodide symporter genes.[12,33,34] Knock-out mice for *PAX8* have a phenotype with hypothyroidism, severely hypoplastic thyroid glands and absence of follicular structures.[17] In humans, reported *PAX8* mutations are R108X (sporadic), R31H (sporadic), L62R (familial), C57Y (familial), Q40P (familial), R31C (familial), S54G (familial), c.989–992delACCC (familial) and S48F (familial). All mutated subjects displayed overt hypothyroidism and, except for one case of coexistence of ectopia and hypoplasia, thyroid hypoplasia was the only reported anatomical feature of TD.[20–26] The majority of mutations are monoallelic and localized to the paired domain and show a severe DNA-binding defect,[20,22,23] suggesting that CH is secondary to *PAX8* haploinsufficiency. Exceptionally, S48F is localized to the paired domain with normal binding affinity and it retains the ability to synergize with *TTF-1*.[26] Also, the reported deletion in exon 7 (i.e. c.989–992delACCC) is located outside the paired domain and retains its binding ability *in vitro*, but it is transcriptionally inactive.[25]

The present study reports mutational analysis of the *PAX8* gene in a large cohort of CH-affected children with dysgenetic (agenesis, ectopia, severe hypoplasia and hemiagenesis) and eutopic thyroid glands. Direct sequencing of the coding region of the *PAX8* gene revealed a known polymorphism (i.e. F329L) in three children with TD and also a new sequence variant, *PAX8*-T225M, in a subject affected with thyroid ectopia and hypothyroidism discovered at 15 years of age and with an unaffected father and sister. Unavailability of grand parents' blood samples restricted us in assessing whether *PAX8*-T225M was *de novo* in the father or an inherited mutation. Evaluation of 240 alleles in normal controls revealed that *PAX8*-T225M does not seem to be a common polymorphism and is probably a rare variant. Functional study of *PAX8*-T225M showed that activation of the *TG* promoter at a level comparable to the activity of *PAX8*-WT was possible and, also, that the *PAX8*-T225M and wild-type had similar synergism with *TTF-1* in transactivation of the *TG* promoter. Furthermore, *PAX8*-T225M, similar to the wild-type, was able to recruit p300 to transactivate the *TG* promoter in the transfected HEK293 cells. However, *PAX8*-T225M did not show significantly increased *TG* promoter activity with 150 and 500 ng of

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p300 with respect to *PAX8*-T225M alone, which is suggestive of a defective synergistic effect of the mutant allele. The cotransfection of *TTF-1*, together with *PAX8*-T225M and p300, totally rescued the synergistic effect of p300 on *PAX8*-T225M-mediated transactivation. These experiments do not provide proof that *PAX8*-T225M participates in the pathogenesis of the disease and certainly it is not the only factor responsible for thyroid ectopia in the proband. The normal phenotype in the father and sister supports the latter assumption. It might be speculated that, unlike *PAX8*-WT, *PAX8*-T225M protein would not function normally in specific concentrations of interactive molecules *in vivo*. In other words, *PAX8*-T225M may require additional contributing factors for production of a TD phenotype. Extreme phenotype variability has also been described[25] for the c.989–992delACCC deletion causing thyroid hypoplasia in the proband and both euthyroid and compensated hypothyroidism in her family, suggesting that other factors may modulate phenotypic expression. Similarly,[22] the patient with *PAX8* mutation Q40P had overt CH with thyroid hypoplasia while the mother, also a carrier of the mutation, had mild hypothyroidism (discovered in adult life, with signs of autoimmunity) and a thyroid of normal size. A polygenic aetiology or stochastic expression of the *PAX* alleles[22,25] has been proposed to explain this variability.

Recently, it has been demonstrated that in the mouse model[11] the combination of partial deficiencies in *TTF-1* and *PAX8* genes results in overt TD which is absent in either of the singly deficient, heterozygous mice. The observed phenotype (small thyroid gland, elevated levels of TSH, reduced TG synthesis) was shown to be strain specific, and the pattern of transmission revealed that at least two other genes, in addition to *PAX8* and *TTF-1*, are necessary to generate this condition. This model strongly suggests that a similar pathogenetic mechanism might be operating in human patients. We speculate that *PAX8*-T225M in our patient might be responsible for the observed phenotype in conjunction with putative alterations of modifier genes, which, together with *PAX8*, control morphogenesis. These genes could be altered in the proband and not in the unaffected father and sister. The normal coding sequence of *TTF-1* in our proband excludes mutations in this gene being responsible for the phenotype but of course we can not exclude alterations of its expression level.

In conclusion, our results further support that *PAX8* mutations in children with CH and dysgenetic or orthotopic thyroid glands are a rare event.

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ORIGINAL ARTICLE

Identification of TSH receptor mutations in three families with resistance to TSH

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Summary

Objective Genetic analysis of the TSH receptor gene in seven subjects with subclinical hypothyroidism (SH), in whom the diagnosis of autoimmune thyroid disease had been excluded by laboratory and instrumental techniques currently available.

Patients Three families where different members (2 children and 5 adults) affected by SH were studied.

Genetic analysis Genomic DNA was extracted from peripheral lymphocytes and the entire coding sequence of the *TSHr* gene was sequenced. *pSVL-TSHr* construct harbouring a Q8fsX62 insertion was obtained by site-directed mutagenesis. COS-7 cells transfected with wild-type and mutant receptor were used for binding studies, flow cytometry, and cyclic AMP (cAMP) determination.

Results A four base pair (bp) duplication in position 41 (41TGCAins), leading to a premature stop of translation at codon 62 (Q8fsX62), was found to be heterozygous in the proband, the father and the sister in Family 1. In Family 2 the proband and the sister were heterozygous for the mutation D410N. In Family 3 the proband and the father were heterozygous for the mutation P162A. After transfection in COS-7 cells, the mutant receptor Q8fsX62 displayed a low expression at the cell surface, and a reduced response to bovine TSH (bTSH) in terms of cAMP production.

Conclusions We identified TSH receptor mutations in seven members of three families with subclinical hypothyroidism.

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Introduction

Thyroid resistance to TSH[1] (RTSH) is a congenital syndrome of variable hyposensitivity to a biologically active TSH molecule.[1] The defect is characterized by elevated serum TSH and normal to very low serum levels of thyroid hormones in the presence of a hypoplastic or a normally sized gland in the proper position in the neck. Depending on the degree of impairment of TSH receptor (*TSHr*) function, subjects can present with euthyroid hyperthyrotrophinaemia at one extreme of the spectrum[1] or severe hypothyroidism at the other extreme.[1,2] Germline loss-of-function mutations of the TSH receptor gene have been described in the case of partial or complete TSH resistance.[3–16] Heterozygotes for the defective allele are usually euthyroid but may have mild hypothyroidism. TSH receptor mutations, however, account for only a minority of cases of RTSH.

In this paper, we studied three families with slight to moderate elevations of circulating TSH and normal free thyroid hormone levels. The diagnosis of autoimmune thyroid disease had been excluded by currently available *in vitro* and *in vivo* tests. In the three families, seven members had isolated hyperthyrotrophinaemia. The entire coding region of the TSH receptor (*TSHr*) gene were sequenced and *TSHr* mutations were found in seven subjects from the three families. The genetic analysis showed a previously described 4 bp duplication in position 41 (41TGCAins)[16] in the proband, her sister and the father in Family 1. A D410N mutation[4] was evidenced in the proband and sister of Family 2. A P162A mutation[1,3] in the proband and his father was found in Family 3.

The functional study revealed that the Q8fsX62 insertion caused an impaired response to bovine TSH after transient expression in eukarytotic cells.

Subjects

Three families with different members affected by isolated hyperthyrotrophinaemia were studied. Serial dilutions of the TSH immunoreactivity were performed in all cases and were always parallel to the standard curve (data not shown). Tests for antithyroperoxidase (TPOAb), antithyroglobulin (TgAb) and anti-TSH-receptor antibodies gave negative results. The ultrasound of the thyroid showed a normal gland in the proper position in the neck with a normoechogenic pattern.

After i.v. TRH challenge the surge of TSH was exaggerated to TSH basal values and production of FT3 was low. Parathyroid function was normal.

The study was approved by the local Ethics Committee and informed consent was obtained from all subjects.

In Family 1 (Fig. 1), the proband (II-1) was a 13-year-old boy, born at term from nonconsanguineous parents. At birth there were

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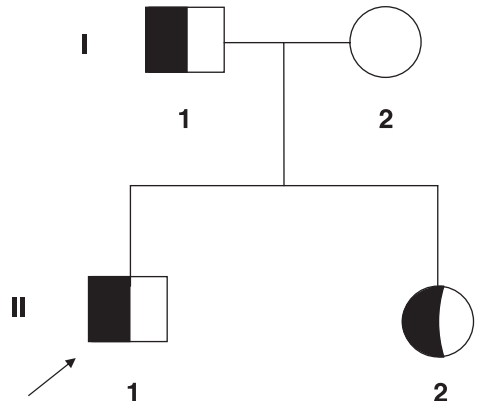

Fig. 1 Pedigree of the members of Family1. Circles, women; boxes, male; arrow, proband; half-closed symbols, heterozygous *TSHr* mutations.

no clinical signs or symptoms of hypothyroidism and neonatal screening revealed a normal spot TSH value. The birth weight was 3·4 kg and height 50 cm. His growth, psychological evolution and education were entirely normal. At 13 years of age he was referred with no specific complaints. He had isolated hyperthyrotrophinaemia [TSH 6·6 mU/l (0·3–3·6); FT4 7·5 pmol/l (7–16)], FT3 3·4 pmol/l (2·5–5·5) with no symptoms or signs of hypothyroidism. A thyroid ecography demonstrated a gland of normal size with a normoechogenic pattern. Peripheral parameters of thyroid hormone action were in the normal range. L-thyroxine (LT4) therapy (50 μg/die) was started and continued for 1 year. Under LT4 therapy TSH was 2·5 mU/l. When he came to our Department for observation LT4 therapy was stopped. After 3 months TSH was 4·0 mU/l (0·3–3·6), FT4 was 8·4 pmol/l (7–16) and FT3 was 4·8 pmol/l (2·5–5·5). His height was 162 cm (90th percentile) and his weight was 60 kg. After TRH challenge the surge of TSH was exaggerated to TSH basal values and production of FT3 was low (Table 1). Parathyroid function was normal.

There was no history of hypothyroidism in the father and the mother of the proband; the father (I-1) had normal free thyroid hormone levels and normal serum thyroid hormones (FT4 10·1 pmol/l; FT3 4·8 pmol/l). His basal TSH level was 3·6 mU/l and he had an absence of antibodies directed against thyroid antigens. The mother (I-2) of the proband had normal thyroid function tests.

The only sister of the proband (II-2) was a clinically euthyroid 9-year-old girl. She had free thyroid hormone levels in the normal range [FT4 9 pmol/l (7–16); FT3 4·3 pmol/l (2·5–5·5)] but her basal TSH level was above the upper limit of the normal range (TSH 6·6 mU/l). Tests for TPOAb, TgAb and anti-TSH-receptor antibodies gave negative results. She had no signs or symptoms of hypothyroidism and some parameters of thyroid status were in the normal range. Her height was 129 cm (50th percentile) and her weight was 34·5 kg.

In Family 2 (pedigree not shown), the proband was a 31-year-old woman who was referred to our Department because of hyperthyrotrophinaemia. She had free thyroid hormone levels in the normal range and a basal TSH level above the upper limit of the normal range [TSH 8·7 mU/l (0·3–3·6); FT4 12 pmol/l (7–16)]. Tests for TPOAb, TgAb and anti-TSH-receptor antibodies gave negative results. Thyroid ultrasound demonstrated a gland of normal size with a normoechogenic pattern. She was treated with LT4 for 10 years and was then referred to us. After stopping therapy for 3 months FT4 was 8·3 pmol/l (7–16), FT3 was 4·2 pmol/l (2·5–5·5) and TSH was 10·1 (0·3–6). After TRH challenge the surge of TSH was exaggerated to TSH basal values and production of FT3 was low (Table 1). Her height was 160 cm and her weight was 59 kg. The sister of the propositus had isolated hyperthyrotrophinaemia (TSH 9·3 mU/l; FT4 9·3 pmol/l; FT3 3·8 pmol/l). The parents were dead and no information was available.

In Family 3 (pedigree not shown), the proband was a 38-year-old man who was referred to our Department because of hyperthyrotrophinaemia. He had free thyroid hormone levels in the normal range and raised basal TSH level [TSH 8·6 mU/l; FT4 8·8 pmol/l (7–16); FT3 4·8 pmol/l (2·5–5·5)]. Tests for TPOAb, TgAb and anti-TSH-receptor antibodies gave negative results. After TRH challenge the surge of TSH was exaggerated to TSH basal values and production of FT3 was low (Table 1). His height was 173 cm and his weight was 78 kg. Thyroid ecography demonstrated a gland of normal size with a normoechogenic pattern. The father had IH with serum TSH levels of 7·8 mU/l and normal serum levels of FT4 and FT3.

The two children of the proband had normal thyroid function tests.

Materials and methods

Laboratory evaluation of thyroid function

Serum free thyroxine (FT4) and free thriiodothyronine (FT3) were measured by radioimmunoassay (FT4 RIA, FT3 RIA, Liso-phase, Laboratori Bouty, Milan, Italy). Thyrotropin (TSH) was assessed

Table 1. Basal and TRH-induced secretion of serum TSH, FT4, FT3 in the proposita of the three families with thyroid resistance to TSH (RTSH)

Propositus		0 min	30 min	120 min	180 min	240 min
II-1 (Family 1)	FT4	8·4	8·3	10·4	9·9	9·6
	FT3	4·8	5	6·3	5·5	5·6
	TSH	4	20·6	7·9	4·5	3·4
II-1 (Family 2)	FT4	8·3	8·2	10·1	10·1	10·3
	FT3	4·2	4·21	5·02	4·66	4·54
	TSH	10·1	47·2	23·2	15·5	12·1
I-1 (Family 3)	FT4	8·8	8·6	9·7	10·2	10·8
	FT3	4·8	4·7	5·33	5·3	5·5
	TSH	8·6	61·1	23·5	15·8	13·2

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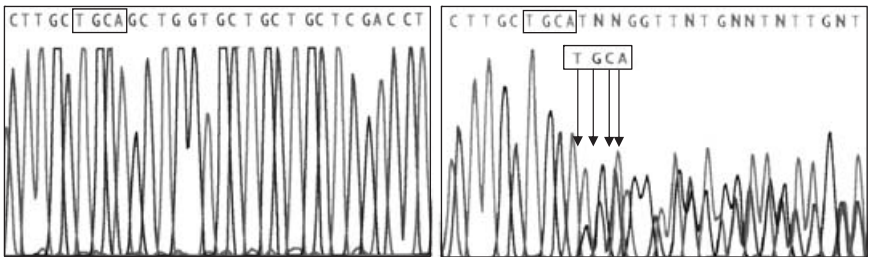

Fig. 2 Nucleotide-sequence traces of *TSHr* of the proband showing the genotype of Family 1. Direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA from peripheral blood demonstrates a 4 bp duplication in position 41 (41TGCAins) leading to predicted frame shift and premature stop of translation at codon 62 (Q8fsX62), almost lacking the full receptor in Family 1. The mutation was heterozygous in the propositus.

with a sensitive method (sensitive-TSH ICMA, Immulite 2000, Diagnostic Products Corporation, Los Angeles, CA). TPOAb and TgAb were measured by IFMA (AIA-PACK TgAb/TPOAb, Tosoh Corporation, Tokyo, Japan). TSH-receptor antibodies (TRAb) were measured using a commercial radioreceptor assay (TRAk human, B.R.A.H.M.S., Berlin, Germany).

Sequence determination

Genomic DNA was extracted from peripheral lymphocytes using standard procedures[7] and after polymerase chain reaction (PCR) amplification *TSHr* gene was sequenced exactly as described previously.[7] In order to confirm the presence of a *TSHr* mutation, the mutation was subcloned in a plasmid and sequences were repeated on individual clones.

Construction and expression of the mutant gene *pSVL-TSHr* construct harbouring 41TGCAins was obtained by site-directed mutagenesis using the GeneTailor site-directed mutagenesis system (Invitrogen Life Technologies, Carlsbad, CA). The accuracy of the recombinant construct was verified by direct sequencing.

COS-7 cells transfected with wild-type and mutant receptor were used for binding studies, flow cytometry and cAMP determination. COS-7 cells were grown in DMEM supplemented with 10% foetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2·5 μg/ml fungizone and 1 mM sodium pyruvate. For the transient expression of wild-type and mutant *TSHr*, COS-7 cells were seeded at a concentration of 150 000 cells/3-cm dish. One day after seeding, the cells were transfected using the DEAE-Dextran method followed by a 2-min 10% dimethylsulfoxide shock.[17]

Forty-eight hours after transfection the cells were used for cAMP production assay, ^{125}I-TSH binding studies and flow cytometry analysis. All experiments were performed in triplicate and each experiment was repeated at least three times. Results were expressed as mean ± standard error (SE). The cAMP assay and binding assay were performed exactly as described previously.[13]

Cell surface detection of TSHr *mutant by microchip flow cytometry analysis*

Cells were detached from culture dishes with 5 mmol/l each of ethylenediamine tetraacetate and ethyleneglycol-bis-(β-aminoethylether)-N, N, N′, N′-tetraacetic acid in phosphate-buffered saline (PBS) and transferred to Falcon tubes (2052, Falcon Labware, Cockeysville, MD). The cells were washed with PBS plus 2% BSA, centrifuged at 500 × *g* at 4 °C for 3 min and stained for 15 min at 37 °C with 0·5 μM calcein (Molecular Probes, Eugene, OR) in a dye loading solution (HBSS, 20 mM Hepes, 1% BSA, Invitrogen Life Technologies, Carlsbad, CA) to specifically dye living cells. Cells were incubated at room temperature for 30 min with the monoclonal antibody BA8 (kindly provided by Dr Costagliola from Brussels, Belgium) directed against the *TSHr* diluted 1 : 10 in PBS plus 2% BSA. To detect the monoclonal antibody the cells were incubated for 30 min at 4 °C in the dark with a goat antimouse IgG Cy5-conjugated (Zymed Laboratories Inc, San Francisco, CA) diluted 1 : 100 in PBS plus 2% BSA. After washing, the cells were resuspended in an appropriate volume of Cell Buffer of the Cell Fluorescence LabChip Kit (Agilent Technologies, Deutschland GmbH, Waldbronn, Germany) to obtain a concentration of 2×10^6 cells/ml, and 10 μl were loaded in Cell Assay Chips according to the manufacturer's protocol. Detection of antibody-stained cells was performed on the Agilent 2100 bioanalyser equipped with the Cell Assay Extension and the Cell Fluorescence software. Data acquisition was performed using the intuitive software package supplied with no requirement to manually set instrument-specific parameters. Results were expressed as a percentage of antibody stained cells within the live population.

Results

The genetic analysis of subjects with isolated hyperthyrotrophinaemia revealed the presence of *TSHr* mutations in seven subjects.

Direct sequencing of exon 1 through 10 of the *TSHr* gene revealed the presence of a previously described anomaly,[16] a 4 bp duplication in position 41 (41TGCAins) leading to predicted frame shift and premature stop at codon 62 Q8fsX62, almost lacking the full receptor (Fig. 2a) in Family 1. The proband (II-1) was heterozygous for the mutation (Fig. 2a). The father (I-1) and the sister were heterozygous for the same insertion (Fig. 2a). The mother of the proband (I-2) had only wtTSHr.

In Family 2, direct sequencing of exon 1 through 10 of the *TSHr* gene revealed the presence of only one anomaly, a GAC/AAC mutation affecting the Asp residue at position 410,[4] which was replaced by an Asn (Fig. 2b). The proband was heterozygous for the mutation (Fig. 2b). The sister (II.2) was heterozygous for this mutation.

In Family 3, direct sequencing of exon 1 through 10 of the *TSHr* gene revealed the presence of a CCT/GCT mutation affecting the proline residue at position 162[3] which was replaced by an alanine (Fig. 2b). A CCC/ACC polymorphism affecting proline at residue 52 (P52T) was also present. The proband was heterozygous for the mutation (Fig. 2b). The father was heterozygous for the same mutation. The two children of the propositus had only wild-type *TSHr* sequences.

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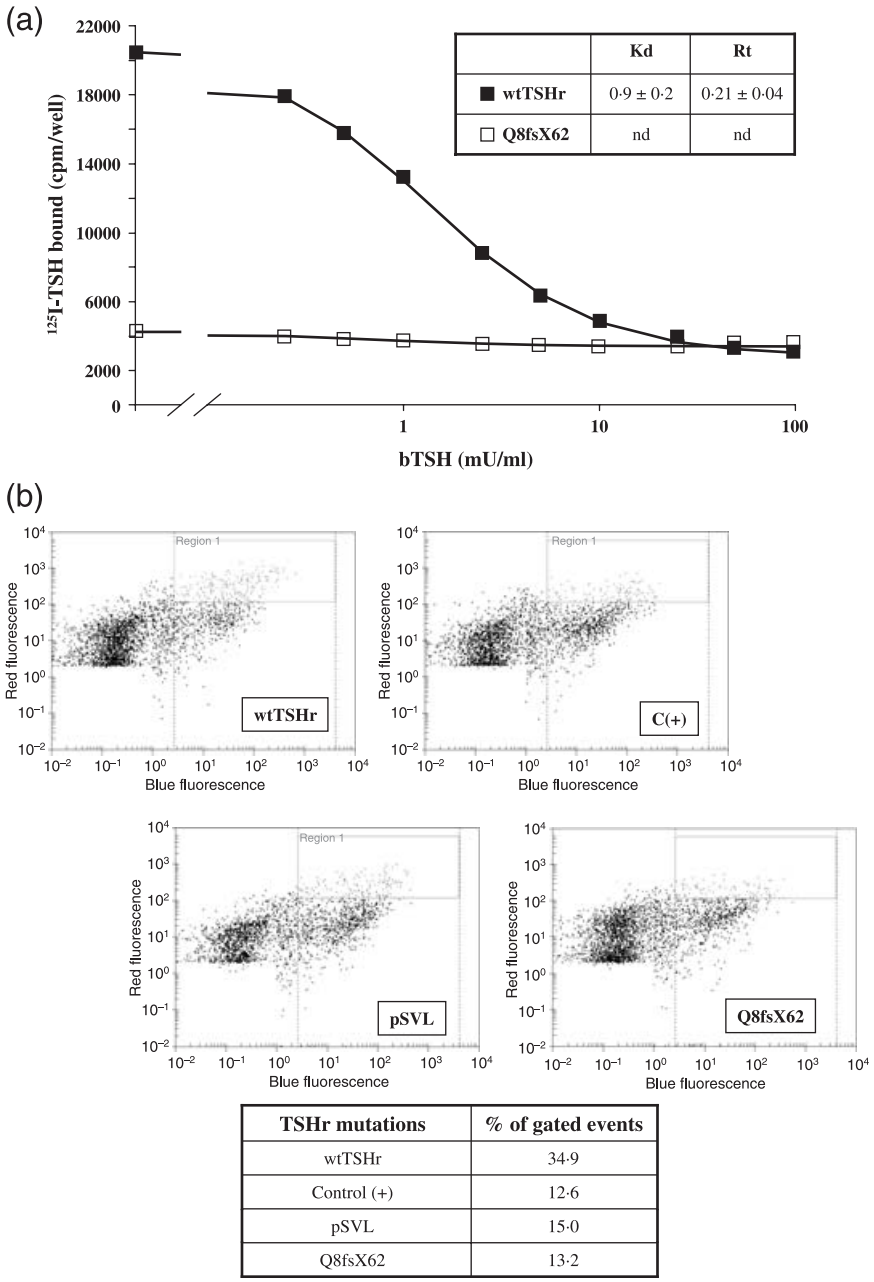

TSHr mutations	% of gated events
wtTSHr	34·9
Control (+)	12·6
pSVL	15·0
Q8fsX62	13·2

Fig. 3 (a) Binding characteristics of the wtTSH receptor and the Q8fsX62 mutant expressed in COS-7 cells. ^{125}I-TSH binding to COS-7 cells transfected with 250 ng/dish of the wtTSHr or ^{41}TGCA^{44} constructs. Total receptor amount (Bmax) (expressed in mU TSH/ml) and kD (also expressed in mU TSH/ml) were computed as described in methods (SE are so small as to fall within the symbols). (b) Expression analysis of the Q8fsX62 mutant by microchip flow cytometry analysis. Results were expressed as a percentage of antibody-stained cells within the live population. Positive control was a known previously described [13] inactivating *TSHr* mutation with a low expression at cell surface (T477I).

The functional characteristics of the mutant receptor Q8fsX62 were studied by transient expression in COS-7 cells. Cells transfected with a cDNA construct encoding the wtTSHr or the empty pSVL vector were used as controls.

Binding studies were performed with a bovine ^{125}I-TSH tracer as described in the section Materials and Methods. The mutant *TSHr* construct showed an extremely low TSH binding capacity, confirming a low level of expression at the cell surface (Fig. 3a). The very low level of expression of the mutant at the cell surface was confirmed by flow cytometry analysis using the BA8 monoclonal antibody directed to the *TSHr* (Fig. 3b).

As previously reported,[5,13] COS-7 cells transfected with wtTSHr exhibited a five-fold increased production of cAMP in the absence of the agonist (180 ± 13 pmol/dish) compared to cells transfected with vector alone (30 ± 8 pmol/dish) (Fig. 4). Cells transfected with the mutant receptor Q8fsX62 showed a lower cAMP production with respect to the wtTSHr but higher than that found in cells transfected with the vector alone (54 ± 13 pmol/dish) (Fig. 4).

The biological response to bovine TSH (bTSH) of COS-7 cells transfected with the cDNA of the different constructs was explored in terms of cAMP accumulation. When challenged with increasing concentrations of bTSH, COS-7 cells transfected with the wtTSHr showed the expected concentration-action response with an EC50 at 0·27 ± 0·02 mU/ml and plateau at 10 mU/ml of bTSH [13] (Fig. 4). Cells transfected with the Q8fsX62 displayed a greatly reduced ability to stimulate cAMP accumulation in response to bTSH.

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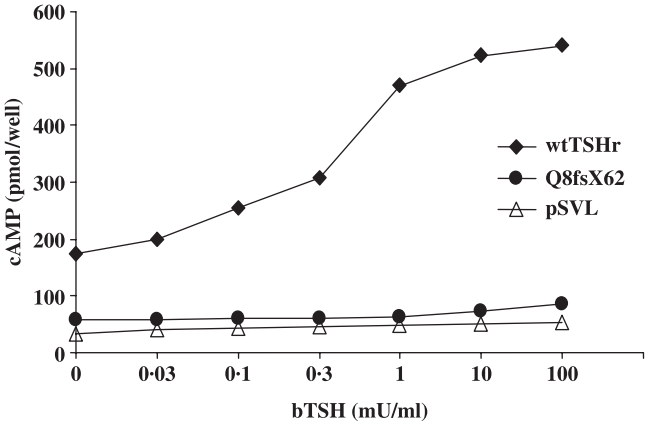

Fig. 4 Functional characteristics of the wtTSH receptor and the Q8fsX62 mutant expressed in COS-7 cells. Stimulation of cAMP accumulation by varying concentrations of bTSH in COS-7 cells transfected with 250 ng of the wtTSHr construct and ^{41}TGCA^{44}. Results in one representative experiment in which triplicate dishes were used are shown as mean ± SE.

Discussion

We describe the clinical and genetic investigation of three families with subclinical hypothyroidism. Subclinical hypothyroidism (SH) is a clinical condition defined as a raised serum TSH associated with normal FT4 and FT3.[18] The patient is usually asymptomatic[18] but careful evaluations have shown consequences on quality of life, cognitive abilities, cholesterol metabolism, heart rate, bone mineral density and atherogenesis.[18,19] Furthermore, SH progresses towards clinically overt hypothyroidism in a substantial number of the patients.[18] Chronic autoimmune thyroiditis is the most frequent cause of subclinical hypothyroidism in adults in western countries[18] and its diagnostic hallmarks are circulating TgAb, TPOAb, or TSH receptor blocking antibodies. A hypoechogenic pattern of the thyroid gland at ultrasound examination is also observed in autoimmune thyroiditis.[20] Serum negative Hashimoto's thyroiditis, although uncommon, has also been described[18] and may be suspected on the basis of a thyroid hypoechogenic pattern at ultrasound[20,21] and then diagnosed by fine needle aspiration of the gland. Autoimmune subclinical hypothyroidism must be distinguished from the rare inherited condition of thyroid resistance to TSH (RTSH). RTSH is a syndrome in which the thyroid shows a variable degree of hyposensitivity to a biologically active TSH molecule.[1] This latter condition may be due to abnormalities in the TSH receptor (*TSHr*),[3–16] PAX-8 transcription factor or a defective Gs-alpha protein (in type 1 pseudohypoparathyroidism).[22] Segregation of this condition in each family we studied followed an autosomal dominant mode of inheritance.

Direct sequencing of the entire coding region of the *TSHr* revealed the presence of *TSHr* mutations in seven subjects included in the study. In Family 1, a 4 bp duplication in position 41 (41TGCAins)leading to predicted frame shift and premature stop of translation at codon 62 Q8fsX62, was found. The proband, the father and the sister were heterozygous for the same insertion. Two previously described inactivating mutations of the *TSHr* gene were identified in the affected members of Family 2 and Family 3. In Family 2, the previously described D410N mutation[4] was identified in the two sisters in the heterozygous state. In Family 3, a previously described inactivating mutation[3] of the *TSHr* (P162A) gene was identified in the proband and the father both in the heterozygous state. The functional study of the D410N described previously[4] showed that this mutant receptor had normal hormone binding properties but a reduced transduction ability showing that the defect was partial. Furthermore, the functional study of the P162A showed a partial impairment of signal transduction.[3] The (41TGCAins) leads to a predicted frame shift and premature stop of translation at codon 62, almost lacking the full receptor. After transient expression in COS cells a very low expression at the cell surface, a low constitutive activity for the adenylyl-cyclase pathway and extremely impaired response to bTSH was shown. The results described in this paper confirm that germline heterozygous *TSHr* mutations may be a cause of SH.

Germline homozygous *TSHr* mutations have been described as a cause of thyroid resistance to TSH. Two missense mutations in the extracellular domain of the *TSHr* were found to produce a condition of partial TSH unresponsiveness with euthyroid hyperthyrotrophinaemia in affected compound heterozygous siblings.[3] Following this initial report, other families with this condition due to compound heterozygous or homozygous inactivating mutations of the *TSHr* gene were described.[4–16] Recently Alberti *et al.*[9] described a higher prevalence of *TSHr* mutations in a series of 10 patients with the phenotype of thyroid resistance to TSH. Several factors contributed to the high prevalence of *TSHr* mutations in this series, because subjects were selected at neonatal screening and familiarity for hyperthyrotrophinaemia was documented in 8 out of 10 subjects. Similarly, Camilot *et al.*[16] reported that among 116 young children with SH, 13 harboured mutations in the *TSHr* gene. In a recent report of our group,[23] inactivating mutations of the *TSHr* gene were identified in two of the three families analysed but none of the 34 sporadic cases of SH. Considering all previous studies and information contained in this paper, we confirm the notion that *TSHr* mutations are probably a rare event in the pathogenesis of sporadic cases of SH without evidence of autoimmunity and the genetic analysis of this gene should be restricted to familial cases of SH.

Furthermore, the patients described in this paper had serum thyroid hormone levels in the low normal range and increased serum TSH levels. In the father of Family 2, the biochemical analysis revealed that his serum TSH was 3·6. Even if a high penetrance has been described, a variable expressivity of RTSH is typical of familial PAX8 and TSH receptor mutations.[1] Mild borderline elevations of TSH were observed in the heterozygous parents of the original family and the other families reported with loss-of-function *TSHr* mutations.[3–16] In particular the D410N mutation was described in a neonate with congenital hypothyroidism and a slightly enlarged eutopic thyroid gland as compound heterozygosity together with Q324X.[4] Contrary to what was observed in our patients, all family members who carried the D410N on a single chromosome had normal serum TSH.[4] The heterozygous P162A was described in five family members with mild borderline elevation of serum TSH.[1] Similar serum TSH levels were measured in the patients described in our study. The molecular mechanisms which are responsible for these observations are not known and might involve haploinsufficiency or dominant negative influence of mutant receptors on wild-type receptor function. Recently Calebiro *et al.*[24] demonstrated that *TSHr*

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mutations are retained in the endoplasmic reticulum probably as a consequence of protein misfolding but maintaining the capability of association with wtTSHr. For this reason wtTSHr is entrapped intracellularly, providing a molecular basis for the dominant form of partial TSH resistance associated with heterozygous mutation in the *TSHr* gene. Similarly, in mice TTF-1 haploinsufficiency produces hypothyroidism mainly through reduction in *TSHr* gene expression which is partially compensated by an increase in serum TSH.[25]

None of the members affected by SH and genetic anomalies of the TSH receptor described in this paper were identified at neonatal screening for congenital hypothyroidism (CH). Although current neonatal screening protocols are highly efficient in the detection of CH, mild yet permanent, hyperthyrotrophinaemia is likely to be missed by screening. We confirm that subtle RTSH may go unnoticed in the neonatal period.

In conclusion, in our study of the three families with SH, genetic alterations of the *TSHr* were identified.

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Sequencing of the entire coding region of the receptor associated protein (RAP) in patients with primary hypothyroidism of unknown origin

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ABSTRACT. The LDL receptor-associated protein (RAP) is involved in secretion of thyroglobulin (Tg) from the thyrocyte to the colloid. Disruption of the RAP gene in mice results in a reduced Tg content within the colloid, leading to subclinical hypothyroidism and histological alterations resembling early goiter. Here we studied the entire coding sequence of RAP in genomic DNA samples from 18 patients with primary hypothyroidism not due to thyroid autoimmunity or dysgenesis. The control group included 21 subjects with no evidence of thyroid alterations. Eleven different polymorphisms with amino-acid substitution and 4 different missense polymorphisms without amino-acid substitution were found in various regions of the RAP gene. Only one polymorphism in exone 7 (V311M) was observed exclusively in patients, but it had been previously reported in normal subjects as well. The remaining polymorphisms were found either both in patients and controls or only in controls and had not been previously reported. The frequency of the various polymorphisms did not differ significantly between patients and controls. Based on these findings, we conclude that alterations of the RAP gene are not a common cause of hypothyroidism, although it cannot be excluded that other, rarer alterations with a pathogenic effect exist, and that they should be investigated in a larger number of patients.
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INTRODUCTION

The LDL receptor-associated protein (RAP) is an endoplasmic reticulum (ER) resident protein that functions as a molecular chaperone for several members of the LDL receptor family (1, 2). In RAP KO mice LDL receptors are retained within the ER and their expression and function on the cell membrane are reduced (1, 2). In thyroid epithelial cells (thyrocytes) RAP is expressed in a TSH-dependent manner and it serves as a molecular chaperone for megalin, an LDL receptor family member responsible for transcytosis of thyroglobulin (3-5). In RAP KO mice expression of megalin on the apical membrane of thyrocytes is reduced, as presumably is its function (6). In addition to megalin, in the thyroid RAP also binds to thyroglobulin (Tg) itself and recent studies have provided evidence that RAP is required for secretion of Tg from the thyrocyte to the colloid (6-8). Thus, in RAP KO mice the content of Tg within follicles is dramatically reduced and Tg is retained intracellularly or secreted by thyrocytes from the basolateral cell membrane (6). In addition, transient transfection of FRTL-5 cells with a RAP construct devoid of the ER retention signal results in impaired Tg release into the medium (8). Whether the effects of RAP on Tg secretion are direct or occur to some extent *via* megalin remains to be established.

The absence of RAP in thyroid epithelial cells results in a mild functional and morphological phenotype, as observed in RAP KO mice (6). In spite of the reduction of follicular Tg, serum levels of thyroid hormones are normal, whereas the TSH is slightly elevated, resembling mild hypothyroidism. Presumably as a consequence of the increased TSH secretion, histological signs of early goiter are present in RAP KO mice. Interestingly, serum Tg levels are markedly elevated, suggesting that Tg is to some extent secreted by thyrocytes from the basolateral cell membrane.

Based on findings in RAP KO mice (6), here we analyzed the entire RAP coding sequence and searched

Key-words: Thyroid, RAP, receptor associated protein, primary hypothyroidsm, thyroglobulin.

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mutations in patients with primary hypothy-
ı in whom the thyroid dysfunction could not be
ducted to thyroid autoimmunity or dysgenesis.

RIALS AND METHODS

ts

d archival genomic DNA samples from 18 patients with hy-
idism and no evidence of thyroid autoimmunity (4 males,
ıles; age range: 14 days-68 yr). Eight patients were found
high TSH levels at neonatal screening and were therefore
ɜed with congenital hypothyroidism. Four patients had a
vhich is why they underwent thyroid assessment and were
o have high serum TSH levels. The remaining 6 patients
dergone thyroid assessment because of mild symptoms
thyroidism. Detailed data on patients are reported in Ta-
he control group included 21 volunteers with no evidence
ıid alterations (4 males, 17 females; age range: 17-55 yr).
informed consent and approval had been obtained from
ɜnts and control subjects when blood samples had been
ncluding approval for storing and using DNA for research
ɜs other than the original one.

ı assays and thyroid assessment

owing measurements were performed in serum samples
patients and controls: free T_4 (FT_4), free T_3 (FT_3) (Lysophase,
. Giovanni, Italy); TSH (Delfia Wallac, Gaithersburg, MD),
(TgAb) and anti-thyroperoxidase (TPO) autoantibodies
ɔ) (Sorin Biomedica, Saluggia, Italy). Tg (Diagnostic Prod-
ɔrporation, Euro/DPC, Gwynedd, UK) was measured in 13
s. Serum assays were performed either at diagnosis or off
roxine (LT_4) therapy. All patients and control subjects un-
ıt an ultrasound examination of the thyroid. Some of the
ɜs also underwent a thyroid scan and some a perchlorate
ion test (Table 1).

t nucleotide sequencing of the RAP gene

lowing 8 pairs of primers, one for each RAP exone, were
ed in the flanking intronic regions:

: forward (5'-CTACAATTCCCAGGAGA-3')
reverse (5'-TCACTTTCCTGCTGCAGAAG-3')
: forward (5'-TTTCTCGCTGTGTCCTGGT-3')
reverse (5'-AATCCGACATCCAAAACACTG-3')
: forward (5'-GCAACCCAGATGTGTTGGC-3')
reverse (5'-TTAACACTCAACGTAACGGCA-3')
: forward (5'-TGGCACTCACGTAGGCAAG-3')
reverse (5'-TGTGTTCCTGAGGGGAGCT-3')
: forward (5'-AGCGAGTTTGAGCTCCCCA-3')
reverse (5'-CGTCGCTACAAGCACCTGC-3')
: forward (5'-ACGCACACTCAGCGTGGTT-3')
reverse (5'-TCCTTACCCGGAAACTGCA-3')
: forward (5'-TCCTTTGGCGTCCTTGCAG-3')
reverse (5'-TGGGGTTCAACTCGGACAG-3')
: forward (5'-AGGCTGAGCTCACAGAGCT-3')
reverse (5'-GCTGCAGTCACCAGAAACAA-3')

ɔlify RAP exons, genomic DNA isolated from whole blood
ɜs was subjected to PCR using the above-mentioned prim-
ɔR was carried out using PCR Master Mix (Promega, Madi-
ı), under standard conditions, namely 35 cycles at 94 C for 40 sec, 1 min annealing at temperatures varying depending on the primer pairs (Exon 1: 58 C; Exons 2, 6 and 8: 55 C; Exon 3: 50 C; Exon 4: 56 C; Exons 5 and 7: 60 C), and at 68 C for 1.30 min. PCR for sequencing was performed using the same primers, as follows: 25 cycles at 96 C for 10 sec, at 50 C for 5 sec and at 60 C for 4 min. Sequencing was performed and analyzed with an ABI PRISM 310 sequencer (Perkin Elmer, Fremont, CA).

Data presentation and statistics

The frequency of RAP mutations or polymorphisms was compared between patients and controls by Chi-squared test with continuity correction, using Stat View (SAS Institute Inc, Cary, NC).

RESULTS

Features of patients

Personal and clinical features of patients are reported in Table 1. Serum TSH at diagnosis or off LT_4 therapy ranged from 3.5 to >100 mU/l. TgAb and TPOAb were undetectable or negative in all cases.

In all children with congenital hypothyroidism (patients 1-8), normal thyroid tissue was seen by neck ultrasound examination, and in those who had undergone a thyroid scan (4 out of 8), a normal uptake of the tracer (99Tc) was detected in the thyroid bed. In addition, in 7 out of 8 children in whom a serum Tg assay was available, Tg was detectable, therefore confirming the presence of thyroid tissue. Only one of these children had a diffuse goiter at ultrasound, whereas in the remaining 7 children the size of the thyroid was normal. A perchlorate dismission test had been performed only in patient 8 and found to be normal.

Of the remaining 10 patients, 4 had a goiter and 6 had a normal thyroid, as found by ultrasound, and in some of them also by thyroid scan. A perchlorate dismission test had been performed in 2 of these 10 patients, showing an organification defect in patient 9.

Sequencing of the RAP gene

As shown in Table 2, 11 different polymorphisms resulting in amino-acid substitutions and 4 different missense polymorphisms with no change in the amino-acid sequence were found. Only one polymorphism in exone 7 (V311M) was found exclusively in patients, but this polymorphism had been reported previously in normal subjects (9). All the remaining polymorphisms were found either both in patients and control subjects or only in control subjects. Two of the missense polymorphisms were found both in patients and control subjects and 2 only in control subjects.

All of the polymorphisms found here had not been reported previously, with the exception of V311M (9). The frequency of the various polymorphisms did not differ significantly between patients and control subjects (Table 2).

Anagraphic, clinical, and serological features of patients. NR: normal range; NA: not available. Serum assays were performed diagnosis or off levothyroxine therapy.

ender	Age	TSH (mU/l) NR: 0.4-3.4	FT_4 (pg/ml) NR: 7-17	FT_3 (pg/ml) NR: 2.7-5.7	TgAb (U/ml) NR: <1-30	TPOAb (U/ml) NR: <1-10	Tg (ng/ml) NR: <1-50	Thyroid ultra-sound	Thyroid scan	Perchlo-rate test
M	14 days	27	9.7	3.9	3	<1	30	Normal	Normal	NA
M	6 months	3.8	10.3	5.2	<1	<1	23	Normal	Normal	NA
F	18 days	10.1	14.6	5.4	<1	<1	113	Normal	NA	NA
M	16 days	69	0.4	<1	<1	<1	34	Normal	NA	NA
F	9 days	20	3.1	1.3	1	<1	19	Diffuse goiter	Diffuse goiter	NA
F	13 days	25.3	5.7	3.5	4	<1	101	Normal	NA	NA
F	27 days	13.7	15.5	6.1	<1	<1	22	Normal	NA	NA
F	22 days	>100	<1	1.9	<1	<1	NA	Normal	Normal	Negative
M	60 yr	37.2	<1	1.7	<1	<1	>3000	Large nodular goiter	Large nodular goiter	Positive
F	4 yr	3.8	10.9	7.2	<1	<1	172	Diffuse goiter	NA	NA
F	68 yr	4.3	11.1	4.2	2	8	NA	Normal	NA	NA
F	16 yr	6.1	6.9	3.6	2	1	7.6	Normal	NA	NA
F	35 yr	4.6	10	2.6	<1	<1	NA	NA	NA	NA
F	25 yr	7.9	10.6	4.4	<1	<1	NA	NA	NA	NA
F	28 yr	5.3	8.7	4.1	<1	<1	NA	Normal	NA	NA
F	55 yr	3.7	10.4	3.4	<1	<1	85	Nodular goiter	Normal	Negative
F	24 yr	5.4	11.5	4.4	1	<1	40	Normal	NA	NA
F	65 yr	3.5	10.7	3.5	<1	<1	65	Nodular goiter	Normal	NA

T_4; FT_3: free T_3; TgAb: anti-thyroglobulin autoantibodies; TPOAb: anti-thyroperoxidase autoantibodies; Tg: thyroglobulin.

ISSION

esent study was undertaken to search for altera-
f the gene encoding the molecular chaperone
patients with primary hypothyroidism not due to
autoimmunity or dysgenesis. Our investigations stemmed from the knowledge that RAP KO mice have a distinct thyroid phenotype, with mild hypothyroidism, high serum Tg levels and histological signs of goiter (6). We sequenced the entire coding region of the RAP gene, which is located on chromosome 4 and com-

- *Polymorphisms of the receptor associated protein gene resulting in amino-acid change (*) and missense polymorphisms not g in amino-acid change.*

Polymorphism	Patient/s no.	Control/s no.	Frequency in patients	Frequency in controls	*p*
N60H (ACC-CAC)*	–	7	0/18 (0%)	1/21 (4.7%)	>0.9999
R113H (CGC-CAC)*	3, 9, 13	5, 13	3/18 (16.6%)	2/21 (9.5%)	0.8438
N114S (AAC-AGC)*	9, 15	11	2/18 (11.1%)	1/21 (4.7%)	0.8772
K180T (AAA-ACA)*	–	5	0/18 (0%)	1/21 (4.7%)	>0.9999
L189L (CTG-TTG)	–	20	0/18 (0%)	1/21 (4.7%)	>0.9999
L193L (CTG-TTG)	–	4	0/18 (0%)	1/21 (4.7%)	>0.9999
L209M (CTG-ATG)*	–	18	0/18 (0%)	1/21 (4.7%)	>0.9999
V210T (ACG-ACC)*	–	18	0/18 (0%)	1/21 (4.7%)	>0.9999
S215N (AGC-AAC)*	8	13	1/18 (5.5%)	1/21 (4.7%)	>0.9999
R220T (AGG-ACG)*	–	15, 17, 18	0/18 (0%)	3/21 (14.2%)	0.2793
T222S (ACG-TCG)*	8	13	1/18 (5.5%)	1/21 (4.7%)	>0.9999
S230I (AGC-ATC)*	4	6, 7, 8, 13, 14, 17, 18	1/18 (5.5%)	7/21 (33.3%)	0.0794
A250A (GCT-GCC)	4, 16, 18	5, 11, 14, 17, 19	3/18 (16.6%)	5/21 (23.8%)	0.8704
S310S (AGC-AGT)	4, 10	21	2/18 (11.1%)	1/21 (4.7%)	0.8772
V311M (GTG-ATG)*	3, 7, 9	–	3/18 (16.6%)	0/21 (0%)	0.1738

8 exons, encoding a protein of 357 amino-
sidues (10). Specific pairs of primers were de-
l in the intronic flanking regions of each exon
ed to sequence the entire gene in 18 patients
control subjects. In spite of the initial hypoth-
at RAP gene alterations may cause a thyroid
type in humans, we did not find any gene al-
ns with a pathogenic effect. Thus, nearly all of
lymorphisms found here were observed both
ents and control subjects, and many of them
control subjects. The only polymorphism that
bserved in just 3 patients (V311M) had been
ed previously in normal subjects (9) and even
we analyzed the frequency of each polymor-
in the two groups, we could not detect any
cally significant difference. Even the fact that
of the patients carried more than one poly-
ism seems meaningless, as similar genotypes
lso seen in control subjects.
ethodology used here allowed for the detection
t mutations and probably of most deletions, but
large or complete deletions of one single allele
utations in the non-coding region of RAP, which
re cannot be excluded based on our data. In ad-
it must be considered that the number of patients
ntrol subjects under investigation was relatively
llowing for the detection of frequent, but not of
ne alterations. Based on this, we can only con-
clude that alterations of the gene encoding RAP are not commonly associated with non-autoimmune primary hypothyroidism, but we cannot exclude that, albeit rarely, they may actually be associated. It may be argued that our series included only a limited number of patients whose thyroid phenotype resembled that of RAP KO mice (6). Thus, only 4 patients had high serum Tg levels and goiter as RAP KO mice (6). In addition, provided RAP gene alterations with a pathogenic effect exist, they would be expected to result in a combined thyroid and kidney phenotype. Thus, RAP KO mice are known to have low molecular weight proteinuria due to a megalin defect in the kidney (11). Because we used archival genomic DNA samples, we could not directly determine whether our patients had kidney alterations. Only 4 patients (no. 9, 16, 17, 18) had undergone a kidney assessment, but there was no evidence of proteinuria nor of other kidney dysfunctions (not shown). In the remaining 14 patients no history of renal diseases was found in their clinical records, but whether they had ever undergone a kidney assessment is unknown. Based on these considerations, it may be speculated that RAP gene alterations with a pathogenic effect might not have been found because the phenotype of our patients was not the one expected in the absence of RAP. In view of these considerations it cannot be excluded that RAP gene alterations with a pathogenic effect may actually exist, which should be investigated in a larger series of patients, although

this will be difficult to achieve because of the rarity of the RAP deficient-like phenotype in humans. The fact that a relatively large number of polymorphisms was found in the RAP gene is in agreement the findings from Van Leuven et al. (9), who also reported several polymorphisms in normal subjects, including one we found here (V311M). To our knowledge, this is the first report on the RAP gene in patients with disease.

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ORIGINAL ARTICLE

Beneficial effects of iodized salt prophylaxis on thyroid volume in an iodine deficient area of southern Italy

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Summary

Objective and subjects Goitre prevalence in school-age children is an indicator of the severity of iodine deficiency disorders (IDD) in an endemic area. The aims of the present study were (i) to provide ultrasound thyroid volume (TV) reference values in a healthy population of school-children aged 11–14 year living in iodine-sufficient areas of Calabria region (ii) to assess both goitre prevalence and urinary iodine (UI) concentration in all children aged 11–14 year from four mildly iodine-deficient areas in which we have carried out a program of salt iodization and (iii) to evaluate the efficacy of the iodoprophylaxis in an adult population living in a small village of the same endemic area.

Design Cross-sectional and prospective studies.

Methods TV was assessed by ultrasonography and iodine intake was estimated by measuring iodine excretion in spot urine samples.

Results We provided the ultrasound normal reference values as a function of age and body surface area, which displayed significant differences from those recommended by the World Health Organization. By adopting local criteria, the prevalence of goitre in children ranged from 23·4% to 27·7% normalized for age and body surface area, respectively, while the UI excretion was < 100 μg/l in 38% of subjects studied. In an adult population living in the same endemic area, goitre prevalence was lowest in the 18–27-year-old age group, and increased progressively with age.

Conclusion We propose for the first time local reference ultrasound values for TV in a population of 11–14-year-old school-children that should be used for monitoring IDDs and have demonstrated the beneficial effects of iodoprophylaxis in consistent with reduced goitre prevalence in children and in the young adult population studied.

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Introduction

Iodine deficiency and related disorders are still a major health concern in most countries of the world. Goitre prevalence and median urinary iodine (UI) concentration in school-age children are currently the two most widely used parameters of iodine deficiency disorders (IDDs). The World Health Organization (WHO) and the International Council for the Control of IDDs (ICCIDD) defined an iodine-deficient area by a median UI excretion < 100 μg/l and classified the goitre endemia by an increase of thyroid volume (TV) that occurs in more than 5% of the school-age population.[1] Although inspection and palpation have traditionally been used for goitre screening, the measurement of TV by ultrasonography is currently the preferred method because of sensitivity and specificity, even if it requires valid reference values obtained from iodine-sufficient populations. WHO/ICCIDD proposed new ultrasound references for TV in school-age children that could be used to define goitre in the context of IDD monitoring.[2] Indeed, some discrepancies between TV reference values reported by different authors[3–7] have suggested the need to establish specific local values in any population with adequate iodine intake.

It is well-demonstrated that iodine prophylaxis has been effective in many countries exhibiting iodine deficiency and is capable of eradicating goitre and cretinism as well as preventing the associated deaf–mutism, short stature and mental deficiency.[8–16] However, in a number of industrialized nations large endemic areas are still present.[17,18]

From our[19] and other[20,21] studies conducted in southern Italy during the early-1990s, the goitre prevalence of school-children was estimated at between 5% and 69% and the mean UI excretion ranged from < 25 to 104 μg/l, indicating a heterogeneity in iodine supply from sufficient to moderate with pockets of severe iodine deficiency among different areas.[19,20] A 2-year program of iodine supplementation in a mild to moderate iodine deficiency area of Calabria resulted in an increased UI excretion together with a decreased goitre

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prevalence, suggesting the usefulness of an effective iodoprophylaxis program in this region.[19]

In the view of previous data, the aim of the present study was first to define normal local ultrasound reference values of TV in 1698 school-children aged 11–14 years from an iodine-sufficient area of southern Italy, and second to assess goitre prevalence in all children living in four villages previously investigated for iodine deficiency[19] and in which, in the last two decades, we have given iodine prophylaxis. Moreover, to evaluate the efficacy of the above-mentioned health program, we estimated goitre prevalence and iodine status in a sample of 707 adults resident in a small village of the same endemic area.

Materials and methods

Subjects

This study was conducted in (i) 1698 healthy school-children (785 boys and 913 girls) aged 11–14 years in iodine-sufficient urban areas of Calabria region; (ii) 209 school-children (90 boys and 119 girls) aged 11–14 years living in four villages of mild to-moderate endemic area in which we carried out a program of salt iodization in the last two decades[19] and (iii) 707 adults from one of four small villages of the same endemic area. Informed written consent was obtained from parents of the minors and from adult subjects. Ethical committees approved the protocol.

Anthropometry

In all school-children we measured body height (centimetres) and body weight (kilograms) from which body surface area (BSA) was estimated using the formula:

$$\text{BSA} = \text{weight (kg)}^{0{\cdot}425} \times \text{height (cm)}^{0{\cdot}725} \times 71{\cdot}84 \times 10^{-4}$$

Thyroid ultrasound

TV was estimated using real-time ultrasound (Logic α100, General & Electrics Instr., Italy) portable instrument, with a 10 MHz linear transducer. Thyroid ultrasound was performed by two independent expert physicians. The subjects were examined in supine position, with the neck hyper extended. School-children's TV was calculated using the formula of a rotation ellipsoid model: width × length × depth × 0·479 for each lobe. Adults' TV was calculated using the formula of a rotation ellipsoid model: width × length × depth × 0·52 for each lobe. Isthmus volume was not taken into account. In the school-children population residing in nonendemic areas, local normal values for thyroid size were assessed; both age and BSA and the upper limit of normality for TV as function of age and BSA were defined by the 97th centile. In endemic areas, goitre prevalence in boys and girls was calculated according to WHO/ICCIDD recommended reference values[2] and to those proposed by the Authors. In an adult population thyroid enlargement was defined as a TV > 18 ml for women and > 25 ml for men, which corresponds to the mean + 3SDs in iodine-sufficient populations as previously reported.[22]

Urinary iodine (UI)

To evaluate the UI, we collected a total of 1408 (808 children and 600 adults) morning spot urinary samples into tubes washed with deionized water and stored at –20 °C until analysis. UI was measured using a manual spectrophotometric method based on the Sandell-Kolthoff reaction, as described by Dunn.[23] Iodine concentration was expressed as μg/l, the iodine deficiency grade was defined according to the WHO's median UI level criteria.[1]

Statistical methods

Statistical analysis was conducted using the Statistical Package for Social Sciences (SPSS, version 15·01). For each subject, the observed measurements carried out by the two examiners were averaged to determine definitive values of TV. The interobserver variation was assessed by repeated analysis of variance (ANOVA), which revealed no statistically significant differences in TV measurements of the two examiners.

The distribution of TV was natural log transformed to assure normality of data. Correlation and multiple regression statistics were used to estimate the association between TV and age and sex. Differences in median TV between groups were evaluated using the Mann–Whitney test.

Pearson correlation was performed for univariate analysis. For each group (i.e. age and BSA), a Gaussian distribution fitted well with mean and standard deviation of the logarithmic values of TV as parameters. Transformed data were then used to compute the age/sex and the BSA/sex specific upper limits (97th percentiles) of normal TV values. Clearly, the analysis involved only subjects with age and BSA included in the respective specified ranges (i.e. age within 11–14 years, BSA within 1·2–1·7 m^2).

Results

Local values for thyroid volume assessed by ultrasound in a school-age population living in a long-term iodine-sufficient area

Totally 1698 children in the 11–14 years age group, born and living in urban iodine-sufficient areas of the Calabria region of southern Italy were recruited to determine local ultrasound reference values for TV. Table 1 shows the features of the studied population. According to the WHO report,[24] iodine sufficiency was proved through the median UI excretion (UIE) which was 125 μg/l. As previously

Table 1. Features (mean ± SD) of the studied population living in an iodine-sufficient area of southern Italy

	Total	Males	Females
Age (year)	12·58 ± 0·9	12·5 ± 0·87	12·6 ± 0·9
Height (cm)	155·81 ± 7·5	155·42 ± 8·23	156·14 ± 6·81
Weight (kg)	49·74 ± 8·6	50 ± 9	49·51 ± 8·24
BSA (m^2)	1·46 ± 0·14	1·46 ± 0·15	1·46 ± 0·13

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Table 2. Ninety-seventh percentile (P97) and median (P50) of thyroid volume measured by ultrasonography as a function of age in a school-age population living in an iodine-sufficient area of southern Italy

		Boys		Girls	
Age (year)	Subjects (*N*)	P50	P97	P50	P97
		ml		ml	
11	193	5·21	9·25	5·87	9·76
12	598	5·37	9·75	5·38	10·03
13	632	5·67	10·70	6·14	10·52
14	275	6·24	11·30	5·90	11·21

Table 3. Ninety-seventh percentile (P97) and median (P50) of thyroid volume measured by ultrasonography as a function of body surface area (BSA) in a school-age population living in an iodine sufficient area of southern Italy

		Boys		Girls	
BSA (m^2)	Subjects (*N*)	P50	P97	P50	P97
		ml		ml	
1·2	134	4·66	8·10	4·68	7·48
1·3	255	4·73	8·68	5·52	8·83
1·4	348	5·46	9·36	5·81	10·01
1·5	466	5·74	9·72	5·90	10·49
1·6	302	6·18	11·20	6·57	10·81
1·7	193	6·88	12·36	7·10	12·10

Table 4. Comparison of goitre prevalence in a school-age population living in an iodine sufficient area of southern Italy, normalized by both body surface area (BSA) and age and calculated using the upper limit values of ultrasound thyroid volume proposed by the World Health Organization (A) and by the Authors (B)

	Goitre prevalence (%)				
	A		B		
Subjects	BSA	Age	BSA	Age	UIE (μg/l)
Total ($n = 1698$)	0·47	1·11	3·35	3·2	Total
M ($n = 785$)	0·63	1·5	3·18	3·2	($n = 628$)
F ($n = 913$)	0·32	0·7	3·5	3·3	125

suggested,[2] the upper limit (97th centile) of normality for TV was calculated as a function of age or BSA, and results are reported in Tables 2 and 3. In the whole population studied, significant differences were observed in goitre prevalence based on ultrasound thyroid reference values adjusted for age and BSA, proposed by the authors and by WHO (Table 4).

Table 5. Features (mean ± SD) of the studied population living in the four villages of southern Italy originally iodine-deficient

	Total	Males	Females
Age (year)	12·95 ± 0·9	13 ± 0·94	12·9 ± 0·86
Height (cm)	156 ± 7·5	157 ± 8·2	155 ± 6·66
Weight (kg)	51·6 ± 9·9	53 ± 9·8	50 ± 9·9
BSA (m^2)	1·54 ± 0·80	1·5 ± 0·15	1·46 ± 0·15

Table 6. Median urinary iodine excretion (UIE) and goitre prevalence in a school-age population living in four villages of southern Italy originally iodine-deficient, normalized by both body surface area (BSA) and age and calculated using the upper limits of ultrasound thyroid volume proposed by the World Health Organization (A) and by the Authors (B)

	Goitre prevalence (%)				
	A		B		
Subjects (*n*)	BSA	Age	BSA	Age	UIE (μg/l)
Total ($n = 209$)	7·1	10·95	27·7	23·4	Total
M ($n = 90$)	6·7	15·55	27·7	25·5	($n = 180$)
F ($n = 119$)	7·56	8·4	27·7	21·84	113

Prevalence of goitre by ultrasound in a school-age population from iodine-deficient area

Our previous endemic goitre survey, conducted in a vast territory of the Calabria region, including four villages (Laino, San Basile, Saracena and Mormanno) located in Cosenza province highlighted a high prevalence of goitre (ranging from 27% to 69% by palpation) with a moderately deficient iodine intake, as demonstrated by a UIE mean (70·7 ± 3·9 μg/l) clearly below the recommended values.[19] In order to gain insights into the effects of a campaign to implement voluntary iodized salt consumption, conducted for two decades, we evaluated, in the same area, ultrasound TV and median UIE in 209 school-children (11–14 year old) whose features are summarized in Table 5. It is worth noting that goitre prevalence, normalized for age and BSA, was 23·4% and 27·7% using our ultrasound local TV references values, while it was 10·95% and 7·1% using WHO criteria (Table 6), with a mean of pathological TV that was 13·25 ± 2·65 ml and 12·44 ± 2·95 ml, normalized for age and BSA. This difference reinforces and justifies the importance of establishing local reference values for TV. Median UIE was 113 μg/l. Out of 180 samples tested 38% (68 children) exhibited individual UI values < 100 μg/l, whereas 8% (14 children) had UI levels below than 50 μg/l. The frequency of UIE for children studied together with those of children from urban iodine sufficient area is shown graphically in Fig. 1. The distribution values of UE did not show significant differences between the two groups. Although most of these children were iodine sufficient at the time of measurement, the thyroid enlargement may reflect mild-to-moderate iodine deficiency that existed in southern Italy regions up to the early 90 s.[19,21]

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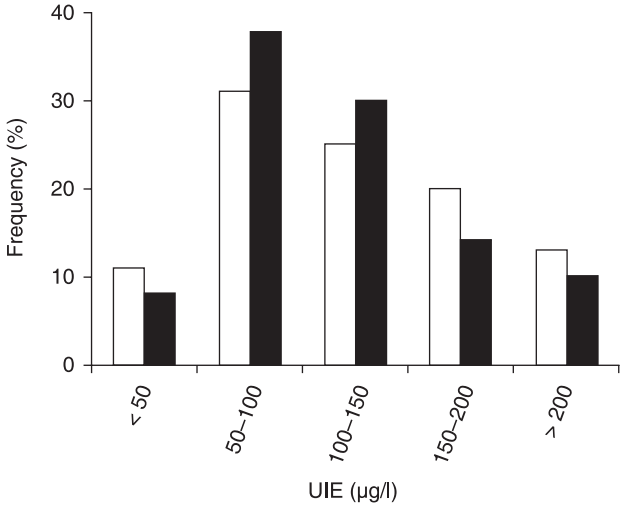

Fig. 1 Frequency distribution values of urinary iodine concentration in a school-age population from iodine sufficient area of southern Italy (□) and from four villages of southern Italy originally iodine-deficient (■).

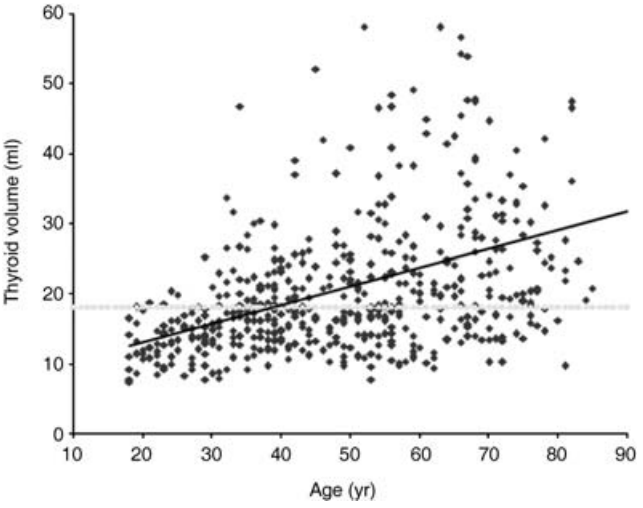

Fig. 3 Thyroid volume in relation to chronological age in females from a village of southern Italy originally iodine-deficient ($R^2 = 0{\cdot}1612$). Regression line through the data is also shown. The horizontal line indicates the upper limit of thyroid volume in females (18 ml).

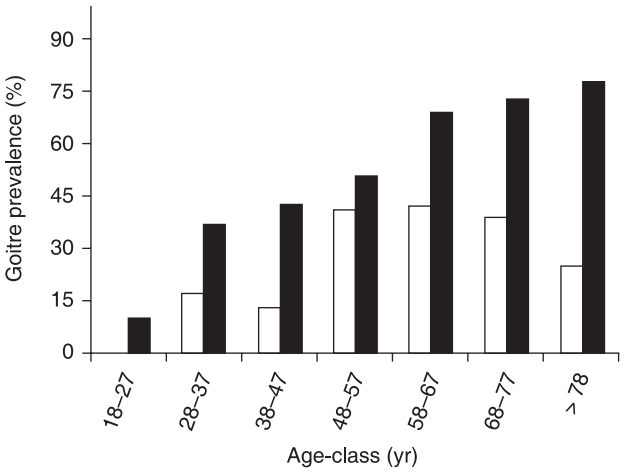

Fig. 2 Goitre prevalence in a adult population according to age-group and grouped in males (□) and females (■).

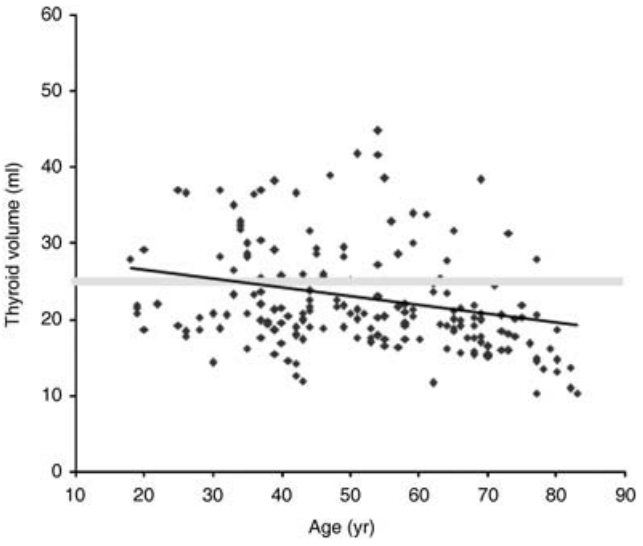

Fig. 4 Thyroid volume in relation to chronological age in males from a village of southern Italy originally iodine-deficient ($R^2 = 0{\cdot}0735$). Regression line through the data is also shown. The horizontal line indicates the upper limit of thyroid volume in males (25 ml).

Trends of ultrasound goitre prevalence in an adult population after long-term iodine prophylaxis

The beneficial effects of iodine prophylaxis was also evaluated in 707 adult people born and living in Laino village where the highest goitre prevalence of southern Italy was previously reported.[19] Figure 2 shows the ultrasound goitre prevalence values in sample evenly grouped according to sexes and age-group. The median UIE value in the entire adult population was 97 µg/l, confirming that the iodine-intake is conspicuously increased in this area due to a persistent iodoprophylaxis campaign. It is interesting to notice how goitre prevalence progressively increased with age in females ($R^2 = 0{\cdot}16$) (Fig. 3), while it appears greatest in middle-aged male groups (Fig. 4). The lowest prevalence of goitre in the youngest age group (Fig. 2) confirms that a population belonging to this age range derives more benefits from iodine supplementation.

Discussion

In the present study, we first established normal local reference values for TV by ultrasound in 11–14-year-old children from an area of southern Italy in which long-standing iodine sufficiency has been documented.[19,20] We observed a significant difference in goitre prevalence between the criteria proposed by the authors with respect to those of the WHO, suggesting the importance of providing local reference values in goitre screening in the context of IDD monitoring in southern Italy. Second, we assessed the iodine status of school-children and an adult population living in an endemic area of Calabria by measuring both UI concentrations and ultrasound TV.

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We observed reduced goitre prevalence in the studied population subjected for two decades to a program of salt iodization, underlying the beneficial effects of sustained iodine prophylaxis.

The WHO has recently estimated the worldwide prevalence of iodine deficiency, which was 36·4% in school-children populations with the lowest prevalence in the Americas (10·1%), where the proportion of households consuming iodized salt is the highest in the world (90%).[25] Surprisingly, the highest prevalence of iodine deficiency is in Europe (59·9%), where the proportion of households consuming iodized salt is the lowest (27%) and, furthermore, most countries have weak or nonexistent national programs.[26]

Thus, universal salt iodization (USI) is the recommended strategy for IDD control and after implementation of USI, careful monitoring of progress towards elimination of IDD is essential. The principal indicator of effect in a population is the median UI, because it is highly sensitive to recent changes in iodine intake.[24] A second indicator is TV as reflected by the goitre prevalence. In endemic areas, although thyroid size decreases in response to increases in iodine intake, TV and goitre prevalence may not return to normal for months or years after correction of iodine deficiency.[18] During this period, goitre prevalence is a poor indicator because it reflects a population's history of iodine nutrition but not its present iodine status.

Many studies have evaluated goitre prevalence by thyroid palpation.[27–30] However, currently, ultrasonography is the most accurate method for the determination of TV even in children who are commonly examined in epidemiological surveys.[31–36] Using this technique in a school-age population, Aghini-Lombardi *et al*. demonstrated a discrepancy in goitre prevalence measured using ultrasound *vs.* that found by palpation,[37] suggesting that thyroid ultrasonography is the more reliable method for precise evaluation of TV in the juvenile population. As measurement of TV is a prerequisite for the accurate estimation of the prevalence of goitre, we adopted ultrasound for the examination of thyroid gland.

Despite recommended upper normal limits of TV calculated according to age or BSA,[2] significant differences exist between countries[7] indicating that local population-specific reference values may be much more reliable than a single international reference. Interestingly, in our study we observed discrepancies in goitre prevalence in a healthy school-age population between our ultrasound local reference values and those proposed by WHO.[2] These findings may reflect genetic features in growth and development as well as environmental factors, including different dietary habits. Moreover, the observed differences may be ascribed to the individual variations commonly seen in the timing of pubertal development, as the influence of somatic growth on thyroid size could be affected by changes in body composition. This supports the utility of providing local reference values to classify the severity of endemic goitre areas.

The data of our survey confirm that iodine prophylaxis, achieved by the recommended use of iodized salt in the daily diet, is able to reduce the development of goitre in children born after such implementation. It is worth noting that we focused our attention on the peripuberal age group (age 11–14 year), in which the effects of iodine deficiency on thyroid development are known to be more evident by ultrasound. Besides, in our previous study conducted on a school-age population ranging from 6 to 12 years the high prevalence of goitre was determined by palpation[19] which is unable to reveal the slight increases in thyroid size. Thus, the score of goitre prevalence evaluated by the latter approach may be underestimated. This gives much more emphasis to the data collected by ultrasonography demonstrating a marked reduction in goitre prevalence in children living in the same endemic area investigated.

However, the beneficial effects of iodine prophylaxis were also observed in the adult population coming from the same endemic area, which displays an almost adequate UI concentration. The youngest adult population investigated (18–27 year) showed almost an absence of thyroid enlargement. On the contrary, the older adult populations, which have mostly lived in a severe iodine deficient environment before beginning iodine supplementation, were less responsive in reducing goitre prevalence. It is of interest to observe that the older age group, showing age-dependency in goitre prevalence, also exhibits gender specificity. For instance, a clear age-dependence is noticed only in females, supporting previous findings that when the iodine need increases, oestrogens might have a positive effect on thyroid follicular cell growth while androgens appear to play an opposite effect, restraining such growth.[38]

In conclusion, the results of this study suggest that in previously iodine deficient populations a higher iodine intake should be required to normalize thyroid volumes, as a low persistent incidence of goitre in school-children population is still observed. Thus, it is essential that a standardized approach should be widely and continuously adopted in line with the legislation of many countries in which mandatory iodine prophylaxis was started many years ago. We recommend the continued use of iodized salt in the daily diet to modify food habits in the entire population as well as its continuous surveillance in the context of IDD monitoring. This is the commitment of the Epidemiologic Observatory of Endemic Goitre and Iodine Prophylaxis of Calabria Region.

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