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Down-regulation of *NR4A1* in follicular thyroid carcinomas is restored following lithium treatment

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SUMMARY

Introduction—The identification of follicular thyroid adenoma-associated transcripts will lead to a better understanding of the events involved in pathogenesis and progression of follicular tumors. Using Serial Analysis of Gene Expression, we identified five genes that are absent in a malignant follicular thyroid carcinoma (FTC) library, but expressed in follicular adenoma (FTA) and normal thyroid libraries.

Methods—*NR4A1*, one of the five genes, was validated in a set of 27 normal thyroid tissues, 10 FTAs and 14 FTCs and three thyroid carcinoma cell lines by real time PCR. *NR4A1* can be transiently increased by a variety of stimuli, including lithium, which is used as adjuvant therapy of thyroid carcinoma with ¹³¹I. We tested if lithium could restore *NR4A1* expression. The expression of other genes potentially involved in the same signaling pathway was tested. To this end, lithium was used at different concentration (10mM or 20mM) and time (2h and 24 h) and the level of expression was tested by quantitative PCR. We next tested if Lithium could affect cell growth and apoptosis.

Results—We observed that *NR4A1* expression was under-expressed in most of the FTCs investigated, compared to expression in normal thyroid tissues and FTAs. We also found a positive correlation between *NR4A1* and *FOSB* gene expression. Lithium induced *NR4A1* and *FOSB* expression, reduced *CCDN1* expression, inhibited cell growth and triggered apoptosis in a FTC cell line.

Conclusions—*NR4A1* is under-expressed in most of FTCs. The loss of expression of both *NR4A1* and the *Wnt* pathway gene *FOSB* was correlated with malignancy. This is consistent with the hypothesis that its loss of expression is part of the transformation process of FTCs, either as a direct or indirect consequence of *Wnt* pathway alterations. Lithium restores *NR4A1* expression, induces apoptosis and reduces cell growth. These findings may explain a possible molecular mechanism of lithium's therapeutic action.

Keywords

Follicular Thyroid Carcinoma; *NR4A1*; *CCND1*; *FOSB* and lithium

INTRODUCTION

The number of thyroid cancer cases is increasing and for women this malignancy has one of the highest rates of increased incidence in the United States. Papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) are the most frequent thyroid cancer subtypes¹. During the last two decades we have seen progress in our understanding of the molecular biology of these cancers. The importance of the MAPK pathway has been identified in PTC, resulting from *RAS/BRAF* mutations or rearrangements of the *RET* oncogene^{2–4}. Less is known about the molecular events underlying FTC.

The most frequent genetic alterations in FTC comprise point mutations of the *RAS* genes and *PAX8-PPAR γ* rearrangements. Although they were initially proposed as potentially associated with pathogenesis of FTC, these genetic events also occur with significant prevalence in FTA^{5–9}. Although it has been suggested that those FTAs with *PAX8-PPAR γ* are more likely to transform into a malignant tumor^{10, 11}, to date there is no compelling *in vitro* or *in vivo* evidence. Moreover, a high proportion of FTCs are negative for the rearrangement⁷ and, therefore, unrevealed genes or pathways may be involved in the pathogenesis of this type of thyroid tumor.

The identification of novel FTA-associated transcripts, could lead to a better understanding of the events involved in pathogenesis and progression of FTC. We compared the gene expression profiles of three SAGE libraries generated from thyroid tumors and a normal thyroid tissue^{12, 13}. The expression analysis was performed originally to locate clinical markers and the data are posted at <http://cgap.nci.nih.gov/SAGE>. Using *SAGE Digital Gene Expression Displayer* (DGED), a tool to compare SAGE libraries, we identified five genes that are expressed in normal thyroid and FTA but not expressed in the malignant FTC. One of these genes, *NR4A1*, encodes an orphan member of the steroid/thyroid/retinoid super-family, whose members act mainly as transcription factors that induce or repress gene expression¹⁴. *NR4A1* (Nuclear receptor subfamily 4, group A, member 1) was of particular interest because it was under-expressed in several tumors compared with the corresponding normal tissues^{15, 16} and because *NR4A1* appears to up-regulate pro-apoptosis genes^{17, 18}.

Consequently, one can hypothesized that lost or reduced *NR4A1* expression in tumors may be associated with malignant transformation of follicular thyroid cells. Although the mechanisms involved in the transcriptional regulation of *NR4A1* is not completely understood, it was described as a target of *Wnt-1* (Wingless-type MMTV integration site family, member 1)¹⁶. The best understood *Wnt* pathway is the canonical pathway, which controls cell proliferation through inhibition of glycogen synthase kinase-3 beta (GSK3 β) and increase in transcriptional activation of β -catenin targets. Although the authors described *NR4A1* as a Wnt-1 responsive gene with decreased expression in tumors, they suggested that *NR4A1* may be up-regulated by a noncanonical Wnt signaling pathway¹⁶.

NR4A1 was also of interest because its expression can be transiently increased by a variety of stimulus, including lithium a drug that has been used to therapy of thyroid cancer^{16, 19–21}. It was shown that lithium acts as a specific inhibitor of the glycogen synthase kinase-3 beta (GSK3 β). Although Lithium has been used as adjuvant to the ¹³¹I therapy of thyroid cancer, the cellular target of lithium is still unknown.

We investigated the expression of *NRA41* in a set of normal thyroid tissues, FTAs and FTCs. Subsequently, we investigated the level of expression of other genes downstream the *Wnt* signaling pathway and correlate their expression with *NRA41* expression. We next sought to test whether lithium could restore the *NRA41* expression in thyroid cell lines and explain a possible molecular mechanism for the effect of lithium as an adjuvant therapy for thyroid cancer.

MATERIAL AND METHODS

SAGE digital gene expression displayer

DGED was used to identify genes differentially expressed between benign (normal tissue and FTA) and malignant (FTC) SAGE libraries. The full set of tag counts for all thyroid libraries is available for downloading or analysis at the Cancer Genome Anatomy Project SAGE Genie Web site (<http://cgap.nci.nih.gov/SAGE>)¹². To identify genes differentially expressed, we defined as criteria $P < 0.05$ and 10-fold difference in the level of expression between the two groups (Fig. 1).

Tissue specimens

A total of 41 thyroid tissue specimens obtained from patients undergoing thyroid surgery for thyroid disease at Hospital São Paulo, Federal University of São Paulo, Brazil, were used in this study. Samples were frozen immediately after surgery and stored at -80°C and included 27 normal thyroids (NTs) 10 FTAs and 14 malignant (FTCs) tissues. Among 14 FTCs, 6 were classified as widely invasive follicular thyroid tumors (WIFC) (capsular and vascular invasion and extension of the tumor beyond the thyroid gland). All tissue samples were obtained with informed consent according to established Human Studies Protocols at Federal University of São Paulo. The study of patient materials was conducted according to the principles expressed in the Declaration of Helsinki.

Cell lines

A FTC (WRO), PTC (NPA) and an undifferentiated thyroid carcinoma (ARO) cell lines used in this study were previously characterized^{22–25}. Cell lines were grown in DMEM (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS (Invitrogen), with 100 units/mL of penicillin and 100 $\mu\text{g/mL}$ streptomycin in a humidified incubator containing 5% CO_2 at 37°C as described^{26, 27}.

RNA extraction and cDNA synthesis and quantitative PCR (qPCR)

Total RNA isolation and cDNA synthesis were performed as previously reported¹³. Briefly, one microgram of total RNA was treated with a DNase (Ambion, Austin, TX) and was reverse-transcribed to cDNA using Super-Script II Reverse Transcriptase kit with an oligo(dT)_{12–18} primer and 10 units of RNase inhibitor (Invitrogen). An aliquot of cDNA was used in 20 μL PCR reaction containing SYBR Green PCR Master Mix and 200 nM of each primer for the target (*NRA41*, *CCDN1* and *FOS*) or reference genes (ribosomal protein S8 and QPC). Primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The PCR reaction was performed for 40 cycles of a 4-step program: 94°C for 15 sec, annealing temperature for 20 sec, 72°C for 20 sec, and a fluorescence-read step for 20 sec. qPCR reactions were performed in triplicate. The threshold cycle (Ct) was obtained using Applied Biosystem software and was averaged ($\text{SD} \leq 1$). Gene expression was normalized using the average of 2-reference genes. Fold changes were calculated as previously described²⁸.

NR4A1, FOSB and CCDN1 expression after lithium treatment

5×10^6 cells WRO cells were plated in 35mm plates. After 24 hours, cells were harvest with fresh medium containing lithium chloride (LiCl; Sigma-Aldrich Corp. St. Louis, MO, USA). Concentration (10mM and 20mM) and time (2 and 24 hours) were determined according to previous studies^{16, 29}. Doses of 10mM and 20mM lithium are commonly used to achieve GSK3 β inhibition and activation of Wnt/ β -catenin signaling²⁹. Total RNA isolation, cDNA synthesis and qPCR for *NR4A1*, *FOSB*, *CCDN1* and reference genes were performed as described above. Additionally, the Sodium-Iodide-Symporter (*NIS*) expression was tested following lithium treatment.

MTT assay

We next investigated the effect of lithium on cell proliferation. In brief, 2×10^4 WRO cells were seeded in 35-mm in quintuplicate, allowed to adhere overnight, and then treated with lithium at 10mM and 20mM concentrations. At each time point, cell growth rates were analyzed following addition of 3,4-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) reagent to the cultured cells as described³⁰. Absorbance was measured using spectrophotometer at a wavelength of 560 nm.

Apoptosis assay

We investigated if lithium could induce apoptosis in WRO cells. Briefly, 2×10^4 cells WRO cells were plated in 35mm plates in quintuplicate. Cells were then treated with lithium at 10mM and 20mM concentrations. At indicated time, Annexin V-positive cells were measured using the Guava Nexin kit as described³⁰.

Statistical analysis

qPCR values were log-transformed before application of statistical tests. Unpaired student t test was used to examine differences between two groups and ANOVA with Bonferroni correction was used to examine differences among groups (StatView software). To test the significance of the relationship between genes, Pearson correlation was used. Chi-squared (X^2) with odds ratio (OR) was performed to determine the risk for tumorigenesis. A *P* value of < 0.05 was considered significant.

RESULTS

Genes potentially associated with pathogenesis of thyroid tumors

Differentially expressed genes can be found by comparison of various SAGE libraries using the SAGE Digital Gene Expression Display (SAGE DGED)³¹. We compared two pools of thyroid SAGE libraries. Pool A included both normal thyroid and FTA and pool B consisted of FTC library. Using $P < 0.05$ as the value for statistical significance and 10-fold as the difference in the level of expression, we identified 6 transcripts differentially expressed between pool A and pool B. Our primary focus was on genes whose expression was lower in FTC library such as *TPO*, *TFF3*, *NR4A1*, and *IYD* (Fig. 1). *NR4A1* was selected for further investigation.

NR4A1 expression in thyroid tissues

The data predicted by SAGE was validated by qPCR using a set of 27 normal thyroid tissues, 10 FTAs and 14 FTCs. We observed that *NR4A1* expression was under-expressed in most FTCs investigated (65%), compared with the expression observed in normals ($P=0.01$) and FTAs (Fig. 2A). The expression was remarkably lower in widely invasive follicular carcinoma than adenoma ($P=0.02$) (Fig. 2B). To determine the risk of tumorigenesis, the level of *NR4A1* expression was analyzed in FTCs (n=14) and normal thyroid (n=22) groups. Using 0.1 as the

cutoff value, expression levels lower than 0.1 were observed in 5 of 22 normal thyroids and 8 of 14 FTCs. A low level of *NR4A1* expression was associated with an increased risk of FTC (Odds ratio= 5.8; χ^2 :6,352; $P = 0.0117$; IC95%: 1.39 – 24.67). To provide a more accurate estimate of the risks of malignancy associated with *NR4A1* level of expression, a larger series and absolute values are needed.

FOSB and CCND1 expression in thyroid samples

Since *NR4A1* was suggested as downstream of Wnt-1 signaling pathway, we investigated the expression of canonical Wnt-1 target genes such as *FOSB* and *CCND1*. Additionally, we sought to determine whether there is a correlation among expression levels for *NR4A1*, *FOSB* and *CCND1*.

FOSB was under-expressed in most of thyroid carcinomas and a number of FTAs compared to normal thyroid, although not significant (Fig. 2C). Intriguingly, *CCND1* was over-expressed in both FTCs ($P < 0.01$) and FTAs ($P = 0.01$), compared to normal thyroid (Fig. 2D).

We found a positive correlation between *NR4A1* and *FOSB* genes in normal tissue ($P = 0.0065$), adenoma ($P = 0.0429$) and carcinoma tissue ($P = 0.0020$) (Fig. 2E). There was no correlation between *NR4A1* and *CCND1* (Fig. 2F).

Lithium restores NR4A1 and FOSB expression and reduced CCND1 expression in FTC cell line

We next investigated expression levels of *NR4A1*, *CCND1* and *FOSB* in thyroid carcinoma cell lines: NPA, ARO and WRO. *NR4A1* and *FOSB* were under-expressed in all cell lines compared to normal thyroid. *CCND1* was over-expressed in WRO, compared to normal thyroid (Fig. 3A, B and C). Since the WRO follicular thyroid carcinoma cell line had patterns expression of the tested genes similar to those observed in FTCs, it was selected for further analysis.

WRO cells were cultured in medium containing Lithium Chloride (LiCl) at different concentrations and time and the level of expression of selected genes were determined. In multiple experiments, qPCR analysis showed a consistent increased of *NR4A1* transcripts in 2h lithium-treated cells compared with untreated controls, in a dose-dependent manner. A greater effect was observed when cells were treated with 20 mM LiCl for 2 h (Fig 4A). After 24 h, *NR4A1* expression was up-regulated only at high doses of lithium (Fig 4B). The expression of *FOSB*, an early gene, was restored after 2 h treatment when 20 mM lithium was used and was down-regulated in cells exposed to 10 mM LiCl for 2 h (Fig. 4C and D). *FOSB* was down-regulated in and 24 h lithium-treated cells compared with untreated controls, in a dose-dependent manner (Fig. 4D).

The expression of *CCND1* was biphasic with a slight increase at 2 h of lithium treatment followed by a down-regulation at 24 h as compared with untreated control cells. Comparable effects of lithium on *CCND1* expression were previously reported³² (Fig. 4E and 4F).

Since lithium has been suggested to act as an adjuvant to ¹³¹I therapy by inhibiting its release from the thyroid, we tested whether the expression of the sodium-iodide symporter (NIS) changed after lithium treatment. We did not find any difference in expression of *NIS* in WRO-treated cells compared with non-treated controls (data not shown).

Lithium inhibits cell growth and induces apoptosis in FTC cell line

Although controversy, previous studies showed that over-expression of *NR4A1* inhibited cell growth and induced apoptosis. Since lithium restores *NR4A1* expression in FTC cell line, we

investigated the effect of lithium on cell growth and apoptosis. As showed in Fig. 5A, lithium treatment resulted in a progressive and dose-dependant cell growth inhibition as compared with the untreated control cells. The reduction in cell proliferation in 20 mM lithium treated cells was statistically significant in all treatment points compared to control cells ($P<0.001$). To quantify the apoptotic rate of WRO cells following treatment, different concentration of lithium were tested at 48 and 72 hours, a time in which growth inhibition was more evident for both concentrations. Lithium significantly increased the number of apoptotic cells in a time and dose-dependent manner ($P<0.001$) (Fig. 5B).

DISCUSSION

In this study, using SAGE Digital Gene Expression Display and validation by qPCR, we found that *NR4A1* was under-expressed in most of FTCs compared to normal thyroid and FTA, and markedly lower in widely invasive follicular thyroid carcinoma.

NR4A1, also known as *Nur77*, *TR3* and *NGFI-B*, an orphan nuclear receptor, was first described as an immediate early gene induced in response to serum or phorbol ester stimulation of quiescent fibroblasts. Although it was initially known for its role in cell proliferation and differentiation, later was shown to be a potent pro-apoptotic molecule in different cell types. Inhibition of *NR4A1* activity either by over-expressing a dominant-negative form or using antisense RNA, inhibited apoptosis^{33, 34}. Consistent with its apoptotic activity, over-expression of *NR4A1* in some cell lines leads to apoptosis. It was demonstrated that its apoptotic activity can be altered through AKT-mediated phosphorylation in the DNA binding domain of *NR4A1* and substantially diminishes its ability to bind DNA and diminish its apoptotic activity³⁵. Others have suggested that *NR4A1* is phosphorylated through the ERK5 pathway³⁶. Moreover, it was suggested that *NR4A1*-mediated apoptosis involves transcription-independent mitochondrial targeting of *NR4A1*. When induced by apoptotic stimuli, *NR4A1* translocates into the cytoplasm where it binds to Bcl2 and provokes conformational change of Bcl2. This results in conversion of Bcl2 from a cell protector to a cell killer^{37,38}.

Based on its apoptotic effects, many studies were performed searching for a link between tumorigenesis and *NR4A1* expression. *NR4A1* expression was reduced in several tumor subtypes compared to normal matched samples¹⁶. Recently it was described that down-regulation of *NR4A1* was a common feature in leukemia blast cells from human acute myeloid leukemia (AML)³⁹. Additional evidence that supports a role for *NR4A1* in tumorigenesis comes from the observation that *NR4A1* abrogation in mice led to a rapidly lethal and transplantable leukemic disease with pathological features most closely resembling AML. Based on these findings, it was suggested a new role of *NR4A1*, a critical tumor suppressor of myeloid leukemogenesis³⁹.

The aforementioned evidences of its potential role as a tumor suppressor gene and the fact that we found a decreased expression of *NR4A1* in follicular thyroid carcinomas compared to normal thyroid and FTA, suggest that the down-regulation of *NR4A1* expression might be an important event during thyroid tumorigenesis.

Given that *NR4A1* induction is regulated by a variety of cell- and stimulus-specific such as growth factors, calcium, inflammatory cytokines, peptide hormones, phorbol esters, neurotransmitters and lithium^{16, 19, 40}, we hypothesize whether these agents could restore the expression of *NR4A1*.

The effect of lithium on thyroid was first observed in patients who received lithium for mood diseases and developed goiter⁴¹. Later it was demonstrated that lithium inhibits the release of radioiodine from the thyroid⁴². Due to this effect lithium is considered an option for adjuvant treatment with radioiodine for selected cases of thyroid cancers^{21, 43, 44}.

Based on the fact that lithium has been used as therapeutic drug for thyroid carcinomas for several years but its therapeutic action remains an enigma and that it induces *NR4A1* expression¹⁶, we hypothesize that lithium might exert its therapeutic effect by restoring the expression of *NR4A1*. We show here that when a follicular thyroid carcinoma cell was exposed to 10 mM or 20 mM of lithium, *NR4A1* expression increased at significant levels after 2 hours of treatment, in a dose-dependent manner.

Additionally, since *NR4A1* over-expression in response to a variety of stimuli and chemotherapeutic agents was involved in growth suppression and apoptosis of many cell types^{18, 45}, we further examined if lithium could trigger apoptosis and reduce cell growth in a FTC cell line. We demonstrated here that lithium inhibited cell growth and induced apoptosis in a dose dependent manner. This helps implicate a potential role for *NR4A1* in thyroid cells and therapeutic mechanism of lithium action. However, we do not know yet if induction of the cell death pathway by lithium is accompanied by translocation of *NR4A1* from the nucleus to the cytosol/mitochondria or by post-transcriptional modification by ERK5 or by AKT-mediated phosphorylation. These possibilities need further investigation.

Converse to our observations, it has been reported that 5 mM lithium stimulates proliferation of two thyroid carcinoma cell lines (papillary and follicular). The authors suggested that inhibition of GSK3 β by lithium lead to an enhancement of proliferation by increasing the β -catenin levels²⁹ and, therefore, mimic the canonical Wnt/ β -catenin signaling^{29,46}. However, it was demonstrated that although the inhibition of the GSK3 β pathway occurred at concentrations as low as 1 mM lithium, substantial growth inhibition of tumor cells does not appear until treatment with 10 mM lithium⁴⁷. Recently, it was demonstrated that treatment with lithium inhibited growth of a medullary thyroid carcinoma cell line (TT) both *in vivo* and *in vitro* and that exerts its effect via inhibition of the glycogen synthase kinase 3 β (GSK3 β). Increased concentration of lithium significantly reduced growth of TT cells in a dose dependent fashion⁴⁸. Inhibition of GSK3 β by specific inhibitors has shown to reduce tumors growth in other cancers including colon, melanoma and glioblastoma^{49–51}. Therefore, these conflicting results could be explained not only for the different concentrations used but also by the fact that the effect of lithium may not be general but cell type specific.

Since it was described that lithium is able to mimic Wnt signaling⁴⁶ it was logical to assume that lithium could modulate other genes downstream in the canonical Wnt/ β -catenin signaling pathway. We therefore, investigated whether lithium modulated the expression of previously described Wnt-1 target genes *CCND1*⁵² and *FOSB*⁵³.

We observed a rapid and dose-dependent increased expression of *FOSB* in thyroid cells following lithium treatment. The rapid and transient expression of *FOSB* parallel those observed for *NR4A1*, suggested they may have a similar mechanism of activation. Our results are in accordance with the literature where a reduced expression of the immediate early gene *FOSB* was observed following stimulation of cells by Wnt-1⁵³. This study also demonstrated that the expression of *FOSB* was significantly reduced in human colon cancer relative to normal tissue⁵³.

We therefore investigated the expression of *FOSB* in the set of samples where *NR4A1* expression was tested. We not only found a reduced expression of *FOSB* in thyroid tumor compared to normal thyroid but a positive correlation with *NR4A1*.

CCND1, a reduced expression of *CCND1* was observed after 24 h treatment with lithium. Kunnimalaiyaan *et al.*,⁴⁸ previously demonstrated a decreased expression of *CCND1* parallel to the increased expression of cell cycle inhibitors such as p21, p15 and p27 following lithium treatment. Whether lithium inhibits GSK3 β and restores *NR4A1* and *FOSB* expression and

decreases *CCND1* expression through the canonical Wnt/ β -catenin signaling pathway, is still not clear.

Although *CCND1* was over-expressed in follicular tumors, there was no correlation with the expression of *NR4A1*. Of note, *CCND1* over-expression and *FOSB* under-expression were previously described as associated with tumorigenesis of papillary thyroid carcinoma^{54, 55}.

Whether *NR4A1* play a role in the pathogenesis of papillary thyroid carcinoma (PTC) is controversy. Previous studies using gene expression profiling following expression of genetic events that are hallmarks of PTC demonstrated that *NR4A1* was among the genes found under-expressed in cells expressing either *RET/PTC3*, *RAS* or *BRAF* genetic alteration, although not confirmed by qPCR⁵⁶. Conflicting evidence is that *NR4A1* was described as one of the genes induced by *RET/PTC3*⁵⁷.

In conclusion, we show that *NR4A1* is down-regulated in follicular thyroid carcinoma compared to normal thyroid and follicular adenomas. This event may be associated with cancer development. The level of *NR4A1* correlates with *FOSB* which suggests they may be part of the same signaling pathway. Lithium restores the expression of *NR4A1* and *FOSB* and reduced expression of *CCND1* which reveals a possible molecular mechanism of lithium action. We suggest that this adjuvant to ¹³¹I therapy modulates *NR4A1* gene expression. Therefore, lithium may be an alternative therapeutic adjuvant mainly in those cases of widely invasive follicular thyroid carcinoma.

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Tag	Gene or Accession	Libraries		Tags		Tag Odds A:B	P
		A	B	A	B		
AGCCTGCGTC	DW444402	1	0	190	0	NaN	0.00
GATGAATAAA	TPO	2	0	134	0	NaN	0.04
CTCCACCCGA	TFF3	2	1	353	2	101.32	0.00
ACACAGCAAG	LOC645221	2	1	165	1	94.64	0.03
TAGTTGAAA	NR4A1	2	1	156	1	89.47	0.04
AAAAGTATTA	IYD	2	1	585	10	33.62	0.00

Total tags in Pool A: 217516
 Total tags in Pool B: 124664
 Total libraries in Pool A: 2
 Total libraries in Pool B: 1
 F (expression factor): 10X
 P (significance filter): .05
 Chromosome: All

Fig. 1. SAGE Digital Gene Expression Displayer (DGED)

The comparison of thyroid SAGE libraries identified those genes differentially expressed genes greater than 10-fold with $P < 0.05$. Pool A includes a normal thyroid library and a follicular thyroid adenoma library. Pool B includes a follicular thyroid carcinoma library.

Fig. 2.

Relative expression (RE) analysis of selected genes in thyroid tumors and normal thyroid. Transcripts levels were normalized to the average of 2-internal controls. The qPCR data were log transformed for statistical analysis. Changes were calculated by comparison of the mean expression value. (A) *NR4A1* was down-regulated in follicular thyroid carcinoma compared to normal thyroid ($P=0.01$). (B) The expression of *NR4A1* was dramatically reduced in widely invasive thyroid carcinoma (WITC) compared to adenoma ($P=0.02$). (C) Although *FOSB* was down-regulated in follicular carcinomas compared to normal thyroid, it was not considered significant (D) *CCND1* was over-expressed in both follicular adenoma ($P=0.01$) and follicular carcinoma ($P=0.006$) compared to normal thyroid. (E) All cases together showing a positive correlation was found between the levels of *NR4A1* and *FOSB*. (F) All cases together, showing

no correlation between *NR4A1* and *CCND1*. Expression was significantly different at the 0.05 level (*).

Fig. 3. Relative levels of expression of *NR4A1* (A), *FOSB* (B) and *CCND1* (C) determined by qPCR in a papillary thyroid carcinoma cell line (NPA), follicular thyroid carcinoma cell line (WRO) and undifferentiated thyroid carcinoma cell line (ARO), compared to the expression (mean) observed in normal thyroid.

Fig. 4.

Effect of lithium treatment on *NR4A1* (A,B) *FOSB* (C,D) and *CCND1* (E,F) expression. A follicular thyroid carcinoma cell line (WRO) was treated with LiCl 10mM (L10) and 20mM (L20) for 2 hours and 24 hours. Transcripts levels were normalized to the average of 2-internal control and qPCR data are averaged from two experiments performed in triplicate. The qPCR data were log transformed for statistical analysis. Changes were calculated by comparison of the mean expression value. Expression was considered significant when $P < 0.05$ (*) and $P < 0.01$ (**).

Fig. 5.

Effect of lithium on cell growth (A) and apoptosis (B) in a follicular thyroid cell line (WRO). The effect was tested using 10mM (L10) and 20mM (L20). (A) Growth assay by MTT was performed as described in material and methods. Lithium significantly reduced cell growth in a dose-dependent manner. The data represents the mean $\pm$ SD of absorbance at 560 nm of experiments performed in quintuplicate on each of four days. (B) To quantify the apoptotic rate of WRO cells following lithium treatment, we analyzed annexin-V binding using a Guava Nexin kit as described in material and methods section. The data represents the mean $\pm$ SD of percentage of apoptotic cells following lithium treatment. An increase of apoptosis was observed in a time and dose-dependent manner. Changes were significant when $P < 0.05$ (*) and $P < 0.001$ (**).