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## Regulation of *14-3-3 $\sigma$* expression in human thyroid carcinoma is epigenetically regulated by aberrant cytosine methylation<sup>\*</sup>

Geeta Lal<sup>a,\*</sup>, Lakshmi Padmanabha<sup>a</sup>, Matthew Provenzano<sup>b</sup>, Matthew Fitzgerald<sup>c</sup>, Jamie Weydert<sup>d</sup>, and Frederick E. Domann<sup>c,e</sup>

<sup>a</sup>Department of Surgery, Division of Surgical Oncology, University of Iowa Hospitals and Clinics, 200 Hawkins Drive, 4641 JCP, Iowa city, IA 52242, USA

<sup>b</sup>Department of Otolaryngology, University of Iowa, Iowa city, IA 52242, USA

<sup>c</sup>Department of Radiation Oncology and Free Radical and Radiation Oncology Program, University of Iowa, Iowa city, IA 52242, USA

<sup>d</sup>Department of Pathology, University of Iowa, Iowa city, IA 52242, USA

<sup>e</sup>The Holden Comprehensive Cancer Center, University of Iowa, Iowa city, IA 52242, USA

### Abstract

Increased *14-3-3 $\sigma$*  expression has been observed by immunohistochemistry in papillary and anaplastic tumors, but not follicular thyroid cancers. *14-3-3 $\sigma$*  mRNA expression and methylation status was examined in tumor cell lines and primary thyroid tissues using real-time RT-PCR, bisulfite sequencing and methylation-specific PCR. Most of the 27 CpG's in the gene's CpG island were methylated in normal thyroid, TPC-1, NPA, FTC-238 and 2–7, which did not express *14-3-3 $\sigma$* . In contrast, they were unmethylated in KAK-1 and anaplastic lines KAT4 and DRO-90. *14-3-3 $\sigma$*  expression was not increased in thyroid carcinomas, the majority of which had a methylated CpG island. In addition, 5-aza-dC treatment increased *14-3-3 $\sigma$*  expression in the FTC-238 and NPA cell lines, which had low baseline expression. We conclude *14-3-3 $\sigma$*  expression in thyroid carcinomas is regulated by CpG island hypermethylation.

### Keywords

*14-3-3 $\sigma$* ; Thyroid; Cancer; Hypermethylation

### 1. Introduction

*14-3-3 $\sigma$*  (stratifin or SFN) is a member of the *14-3-3* family of proteins which play critical roles in signal transduction pathways and cell cycle regulation [1]. *14-3-3 $\sigma$*  was initially characterized as a human mammary epithelium-specific marker 1 (HME1) and functions as a negative regulator of cyclin-dependent kinases [2,3]. *14-3-3 $\sigma$*  is the only isoform which is induced following DNA damage [4]. Increased *14-3-3 $\sigma$*  levels lead to G2/M arrest via sequestration of cyclinB1-cdc2 complexes in the cytoplasm [2]. Furthermore, this protein may also be involved in G1/S transition via binding to CDK2 and CDK *in vitro* [3].

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\*Corresponding author. Tel.: +1 319 356 1727; fax: +1 319 356 1218. E-mail address: E-mail: Geeta-lal@uiowa.edu(G. Lal).

Previous work from our laboratory shows that *14-3-3 $\sigma$*  expression is tissue specific [5]. Certain normal tissues such as skin, mouth, prostate, airway and breast express *14-3-3 $\sigma$* , whereas other tissues have undetectable levels of this protein. Experiments also demonstrated that *14-3-3 $\sigma$*  expression in these tissues is linked to an unmethylated CpG island, whereas loss of *14-3-3 $\sigma$*  expression is associated with its hypermethylation.

*14-3-3 $\sigma$*  also has an important role in carcinogenesis and is lost in several tumor types. Nacht et al. reported that *14-3-3 $\sigma$*  expression was down-regulated in breast cancer cell lines and primary breast carcinomas [6,7]. These investigators also noted that loss of heterozygosity (LOH) and intragenic mutations were not major contributing mechanisms, rather *14-3-3 $\sigma$*  was epigenetically silenced by hyper-methylation of a discrete CpG rich region. Demethylation of the gene coincided with re-expression of *14-3-3 $\sigma$*  mRNA in cell lines which had lost expression of this gene.

Loss of *14-3-3 $\sigma$*  expression in association with hypermethylation has also been reported in several other cancers, including ovarian [8], hepatocellular [9], prostate [10], some lung [11], gastric [12] and endometrial carcinomas [13]. It is important to point out that this is not the only mechanism for *14-3-3 $\sigma$*  down-regulation. In some breast cancer and prostate cancer cell lines, loss of *14-3-3 $\sigma$*  has been shown to occur at the post-translational level and appears to be mediated by proteasome directed protein degradation [10,14].

Interestingly, in some tumor types, *14-3-3 $\sigma$*  expression appears to be increased rather than lost. Higher *14-3-3 $\sigma$*  expression (by immunohistochemistry) was noted in lung cancers when compared to normal lung tissues [15]. *14-3-3 $\sigma$*  expression has also been reported to be increased in head and neck squamous cell carcinomas [16] and pancreatic cancers by microarray and real-time PCR studies [17], when compared to corresponding normal tissues. Perathoner et al. [18] also demonstrated strong *14-3-3 $\sigma$*  expression by Western blot analysis in 4/8 colorectal carcinoma cell lines and 6/8 tumor tissue pairs. *14-3-3 $\sigma$*  was also overexpressed by immunohistochemistry in 39% of colorectal carcinomas and appeared to be associated with a worse prognosis. Overexpression in this tumor type also appeared to be epigenetically controlled by demethylation of the CpG island [19].

Many genes important in thyroid carcinogenesis have been shown to be inactivated by promoter hypermethylation, including *p16* [20], thyroid-stimulating hormone receptor gene (*TSH-R*), *SLC26A4* (Pendred syndrome gene) [21,22] to name a few. Exposure to external beam radiation is one of the most important risk factors for thyroid carcinoma development. It can therefore be hypothesized that *14-3-3 $\sigma$* , which is upregulated in response to DNA damage, has an important role in thyroid carcinogenesis. In fact, Ito et al. [23,24] using immunohistochemistry and Western blot analyses, have demonstrated that *14-3-3 $\sigma$*  expression is increased in papillary and follicular variant of papillary thyroid tumors, however, the protein does not appear to be expressed in follicular thyroid cancers. Highest levels of expression were noted in anaplastic thyroid cancers [23]. The authors also hypothesized that *14-3-3 $\sigma$*  may contribute to the dedifferentiation of thyroid carcinoma cells since increased protein expression was noted in higher stage papillary thyroid carcinomas and anaplastic thyroid cancers. The mechanism of this variable *14-3-3 $\sigma$*  expression in thyroid tumors has not been previously studied. We hypothesized that *14-3-3 $\sigma$*  expression in thyroid carcinoma is epigenetically regulated by aberrant methylation of its CpG-rich island.

## 2. Materials and methods

### 2.1. Cell culture and thyroid tissues

Thyroid tumor cell lines studied included KAK-1 (derived from a follicular adenoma), TPC-1, KAT10, NPA (Papillary carcinoma), FTC-133, FTC-236, FTC-238 (follicular carcinomas, the

latter two represent lines derived from a lymph node metastasis and a lung metastasis from the same patient), XTC-1 (Hurthle cell carcinoma), 2-7 (poorly differentiated thyroid cancer), DRO-90 and KAT4 (both anaplastic carcinoma). The cell lines were maintained in Glutamine plus DME H-21:F-12 media (Fisher#MT-15-090-CM) containing 10% FBS, 10 µg/ml Insulin, 0.01 U/ml TSH (Sigma# T-8931), 250 ng/ml Fungizone, 50 µg/ml Penicillin. DRO-90 was maintained in RPMI 1640 with L-glutamine. The cells were incubated to 80–90% confluence prior to total RNA extraction. Airway epithelial cells, which are known to express high levels of the 14-3-3σ transcript [5] were used as a positive control.

Normal thyroid tissues and tumor samples were obtained from the Tissue Procurement Core at the University of Iowa Hospitals and Clinics. Tissues were obtained in accordance with a protocol approved by the Institutional Review Board for Human subjects. The tissues were harvested and snap frozen in liquid nitrogen at the time of thyroidectomy. The samples were stored at –80 °C until total RNA extraction. Diagnoses of banked specimens were confirmed by histological examination.

## 2.2. Drug treatment

Cell lines were grown for 5–7 days in the presence or absence of 1 µM of 5-aza-2'-deoxycytidine, a known DNA methyltransferase inhibitor (Sigma, St. Louis, MO), and then analyzed for 14-3-3σ mRNA expression by real-time PCR, as described below. The duration of drug treatment was selected based on the growth patterns of this cell line and previous studies from our lab using other thyroid cancer cell lines [25].

## 2.3. RNA extraction and cDNA synthesis

Total RNA was extracted after homogenization of cells and tissues using TRIzol (Invitrogen, Carlsbad, CA), followed by DNAase digestion (RNAase free DNA-ase set, Qiagen, Valencia, CA) and on-column clean up with the RNeasy MinElute kit (Qiagen, Valencia, CA). Total RNA (2 µg) was reverse transcribed with the Superscript II RNAase H-reverse transcriptase kit (Invitrogen, Carlsbad, CA) for cDNA synthesis using random hexamer primers. The cDNA reaction was diluted to 1:10 for use as template for real-time RT-PCR.

## 2.4. Real-time RT-PCR

14-3-3σ primers and probes were obtained from Applied Biosystems (Assays on Demand kit®, HS00602835\_s1 SFN Applied Biosystems, Foster city, CA). 18S ribosomal primers and probes (Applied Biosystems, Foster city, CA) were used as internal controls for the expression analyses. PCR amplifications were performed in a final reaction volume of 50 µl containing 25 µl of Taqman Universal PCR Master mix (Applied biosystems, Foster City, CA) and 2.5 µl of the primers and probe mix, as per the manufacturers' instructions. All reactions were carried out in the ABI PRISM® 7700 Sequence detector Thermocycler (Applied Biosystems, Foster city, CA). The  $\delta C_t$  for 14-3-3σ mRNA expression was calculated relative to the  $C_t$  (threshold cycle) of 18S ribosomal RNA. All reactions were performed in duplicate and relative mRNA expression was calculated using the formula  $2^{(-\delta\delta C_t)}$ , as previously described [26]. Appropriate positive and negative controls were used for PCR analyses.

## 2.5. Immunohistochemistry

Immunohistochemical staining was performed on formalin fixed paraffin embedded tissue sections using a polyclonal antibody against 14-3-3σ (Abcam, Cambridge, MA). Paraffin sections were deparaffinized in xylenes, rehydrated using graded alcohols, and rinsed in distilled water. Antigen retrieval was performed using citrate buffer, pH 6.0, and two cycles of microwave heating. After slides were cooled to room temperature, endogenous biotin was quenched with 3% H<sub>2</sub>O<sub>2</sub>. Sections were covered with primary antisera (14-3-3σ, rabbit,

polyclonal, 1:4000 dilution), incubated overnight at 4 °C, and rinsed. Sections were then covered with secondary antisera (anti-rabbit, Envision+System Labeled Polymer-HRP, Dako, Carpinteria, CA) and incubated 30 min at room temperature and rinsed. Visualization was performed with Dako Liquid DAB+substrate chromogen, and counterstaining was performed with Harris formula hematoxylin. Negative control slides were prepared by substituting non-immune rabbit IgG for the primary antibody. All the cases were evaluated by a single pathologist (JW) who was blinded to the results of the real-time RT-PCR. The scoring system was based on extent of staining, not intensity of staining: No staining = 0, < 25% cells positive = 1+, 25–75% cells positive = 2+ and >75% cells positive = 3+.

## 2.6. DNA extraction and methylation analysis

The methylation status of the *14-3-3 $\sigma$*  CpG island was examined using sodium bisulfite genomic sequencing, as previously reported by our lab [5]. Briefly, genomic DNA was isolated using the DNAeasy Tissue kit (Qiagen, Valencia, CA) and modified with sodium bisulfite using EZ-DNA methylation kit (Zymo research, Orange, CA). Nested PCR was performed using previously published primers for the *14-3-3 $\sigma$*  CpG island [5]. The first round of PCR was performed with 2.4  $\mu$ L of modified DNA in a total reaction volume of 25  $\mu$ L. PCR conditions were as follows: 95 °C for 5 min, 35 cycles of 92 °C for 2 min, 58 deg:C for 3 min, 72 °C for 2 min and a final extension of 72 °C for 5 min. In the second round of PCR, 2.5  $\mu$ L of the first PCR product was amplified as indicated above, with the exception of an annealing temperature of 60 °C.

The PCR product was electrophoresed on a 2% agarose gel, followed by gel extraction and cloning into a TOPO TA vector according to the manufacturer's instructions (TOPO TA Cloning Kit, Invitrogen, Carlsbad, CA, USA). Five to ten positive recombinants were isolated using a Qiaprep Spin Plasmid Miniprep kit (Qiagen, Valencia, CA) and sequenced on an ABI automated DNA sequencer. The methylation status of the individual CpG sites was determined by comparison of the sequence obtained with the known *14-3-3 $\sigma$*  sequence. The number of methylated CpGs at a specific site was divided by the number of the clones analyzed to calculate the percentage methylation at each CpG site. For calculation of the total or overall promoter methylation frequency, the total of all the CpG sites that were methylated from all the clones was counted and divided by the total number of CpG sites. Bisulfite-treated DNA was also used for methylation-specific PCR using previously published primer sets [13] to distinguish between methylated and unmethylated DNA in both tumor cell lines and primary thyroid tissues and tumors.

## 2.7. Statistical analysis

The SPSS software was used to perform the statistical analyses. All statistical tests were two-sided and performed at the 5% level of significance.

## 3. Results

### 3.1. *14-3-3 $\sigma$* mRNA expression in tumor cell lines and thyroid tissues

As displayed in Fig. 1(A), The *14-3-3 $\sigma$*  transcript was undetectable by real-time RT-PCR in, all the follicular carcinoma-derived cell lines and the poorly differentiated cell lines 2–7. Very low levels of transcript were detectable in the papillary and well-differentiated cell lines (TPC-1, NPA and KAT-10) and the Hurthle cell cancer line XTC-1. Markedly increased *14-3-3 $\sigma$*  mRNA levels were noted in KAK-1 and the anaplastic carcinoma cell lines KAT-4 and DRO-90. In total, 3/11 (28%) of cell lines demonstrated *14-3-3 $\sigma$*  upregulation.

*14-3-3 $\sigma$*  expression was also examined in 36 primary thyroid tissues (4 normals, 14 papillary thyroid cancers, 10 follicular variant of papillary carcinomas, 4 follicular thyroid cancers, 3

Hurthle cell cancers and 1 anaplastic cancer) by real-time RT-PCR. The *14-3-3 $\sigma$*  transcript was undetectable in all normal thyroid tissues and tumor types examined as shown in Fig. 1B.

### 3.2. 14-3-3 $\sigma$ methylation status in thyroid cell lines and tissues

Bisulfite sequencing was performed in 7 of the 11 studied cell lines, in addition to normal thyroid tissues. Fig. 2 and Fig. 3 illustrate the methylation frequency and distribution at each of the CpG sites among the sequenced amplicons. The majority of the 27 CpGs investigated were methylated in normal thyroid tissues and in TPC-1, NPA, FTC-238 and 2-7, all of which did not express *14-3-3 $\sigma$* . In contrast, the CpGs were largely unmethylated in KAK-1 and KAT4; and DRO-90 appeared to contain a mixed population of cells with 4/10 methylated clones. All of these cell lines expressed abundant *14-3-3 $\sigma$*  transcript. Overall methylation frequency of the *14-3-3 $\sigma$*  CpG island in the various cell lines is depicted in Table 1. The total methylation frequency in cell lines expressing *14-3-3 $\sigma$*  ranged from 2% to 34% and from 73% to 94% in cell lines expressing little to no transcript. This difference was statistically significant, (*t*-test, *p* < 0.01).

Methylation-specific PCR was performed on DNA derived from the tissues examined by real-time RT-PCR, as shown in Fig. 4. All 4/4 (100%) normal thyroid tissues were methylated and did not express *14-3-3 $\sigma$* . Of the 32 tumors, bright unmethylated bands were noted in 2 Papillary cancers and 3 Follicular variant of papillary cancers, for an overall methylation rate of 27/32 or 84%. There was no statistical difference in the methylation rate between tumors and normal tissues (Fisher's exact test). The 5 tumors with the unmethylated transcript also had undetectable *14-3-3 $\sigma$*  expression.

### 3.3. 5-aza-dC treatment and 14-3-3 $\sigma$ reactivation

The real-time RT-PCR and sequencing data suggest an association between cytosine methylation and *14-3-3 $\sigma$*  expression. To test whether CpG methylation in *14-3-3 $\sigma$*  was functionally linked to its silencing in thyroid cell lines, we attempted to induce *14-3-3 $\sigma$*  expression in the *14-3-3 $\sigma$*  negative FTC-238 and NPA cell lines, by treatment with 5-aza-dC. This treatment resulted in significantly increased *14-3-3 $\sigma$*  mRNA expression, as shown in Fig. 5. This was accompanied by demethylation of the CpG island, as examined by methylation-specific PCR.

### 3.4. 14-3-3 $\sigma$ expression by immunohistochemistry

Due to the discrepancy in our real-time RT-PCR data and published expression data [23] in primary thyroid tumors, *14-3-3 $\sigma$*  expression was also examined by immunohistochemistry in 21 available archived tumors corresponding to the above specimens. The results are shown in Table 2 and Fig. 6. Squamous cell carcinoma was used as a positive control. *14-3-3 $\sigma$*  staining was observed primarily in the cytoplasm. None of the normal tissues showed *14-3-3 $\sigma$*  expression. Staining was noted in majority of papillary cancers and follicular variant of papillary cancers. Faint staining was noted in 1 follicular cancer, 1 Hurthle cell cancer, and the only anaplastic cancer that was analyzed. However, the pattern of staining was highly variable between the tumors.

## 4. Discussion

Although *14-3-3 $\sigma$*  expression has been previously examined in thyroid tumors and normal tissues using immunohistochemistry,[23] the mechanism of its regulation has not been studied. In the present study, we have used a panel of thyroid tumor cell lines and primary tumors to determine if aberrant methylation of the gene's CpG island is responsible for the regulation of *14-3-3 $\sigma$*  expression. Our experiments confirm that normal thyroid tissues do not express *14-3-3 $\sigma$* , as detected by real-time RT-PCR. In the previous studies of thyroid tumors [23,27],

14-3-3 $\sigma$  expression was shown to be increased, rather than lost, in papillary thyroid and follicular variant of papillary carcinomas, with highest expression levels in poorly differentiated and ana-plastic tumors. Interestingly, primary follicular thyroid carcinomas did not appear to overexpress 14-3-3 $\sigma$  [23,27]. Our work in cell lines also reflects this to some extent, in that low levels of 14-3-3 $\sigma$  transcript were detected in well-differentiated cell lines, with highest levels seen in the anaplastic cancer-derived cell lines. Furthermore, as in the previous work, the follicular cancer-derived cell lines showed negligible 14-3-3 $\sigma$  expression. The increased 14-3-3 $\sigma$  expression seen in the KAK-1 cell line is intriguing, given that it is derived from a follicular adenoma. A review of the literature demonstrates that KAK-1 appears to be morphologically similar to other thyroid carcinoma-derived cell lines such as KAT-4 and KAT-10 [28]. In addition, it seems to have lost expression of several genes, similar to other thyroid carcinoma cell lines [25,29–31]. In this regard, KAK-1 may behave more like a malignant rather than a benign tumor-derived cell line in culture.

Per our experiments, increased 14-3-3 $\sigma$  expression does not appear to be a frequent event in primary thyroid cancers. The 14-3-3 $\sigma$  transcript was undetectable in all the 4 normal and 32 primary thyroid cancers examined, including papillary and ana-plastic tumors. This is in marked contrast to previously published data [23] in which 82/82 papillary cancers and 21/23 anaplastic cancers were reported to express 14-3-3 $\sigma$  by immunohistochemistry. Studies have shown that real-time RT-PCR is a reliable technique for the quantification of gene expression [32,33]. In general, the use of immuno-histochemistry as a test of gene expression tends to be significantly affected by the sensitivity and specificity of the antibodies used (polyclonal vs. monoclonal) and the type of tissues examined (frozen vs. formalin fixed). In addition, immunohistochemistry results can vary depending upon different scoring and interpretive criteria used to evaluate the cases [34]. In order to minimize these effects, our immunohistochemistry analyses were performed in a single lab, with the results being scored independently by a single pathologist (JW). Antibody to 14-3-3 $\sigma$  is commercially available and we have used a rabbit polyclonal antibody, similar to the published studies. Staining was observed in the majority of papillary cancers and follicular variant of papillary cancers, with little staining in follicular cancers. This is similar to previous work, however, the results were discrepant with those obtained by real-time RT-PCR. It is important to note that polyclonal antibodies are not always optimal for immunohistochemical analyses. The pattern of staining observed also suggests that these results are more likely to represent non-specific binding, and that the true expression is reflected by the real-time RT-PCR results.

Our studies also show that the expression of 14-3-3 $\sigma$  in thyroid tumor cell lines and primary thyroid tissues and tumors is linked to the methylation status of the 14-3-3 $\sigma$  CpG island. As judged by bisulfite sequencing, high overall methylation frequencies were seen in cell lines that had low levels of 14-3-3 $\sigma$  expression. In contrast, cell lines showing appreciable expression had low overall methylation frequencies in the 2–3% range. DRO-90 demonstrated a mixed population of cells with an overall methylation frequency of 34%, i.e. the CpG islands were largely unmethylated in this cell line. With respect to primary thyroid tissues, the methylated form of the 14-3-3 $\sigma$  CpG island was detected in all tumor and normal samples. This is not an unexpected finding as the tissue samples were obtained macroscopically rather than microdissected, and thus can be expected to contain both normal and neoplastic tissue. However, a bright band corresponding to the unmethylated product was also observed in 5 (15%) tumors which did not express the transcript. This may be due to the fact that out specimens were not microdissected. Alternatively, it suggests that 14-3-3 $\sigma$  expression in a small proportion of these tumors may be regulated by a different mechanism.

This association between gene expression and methylation status was further confirmed by the induction of 14-3-3 $\sigma$  expression in the FTC-238 and NPA cell lines after treatment with the

DNA methyltransferase inhibitor 5-aza-dC. Demethylation of the CpG island as evidenced by MS-PCR was accompanied by upregulation of 14-3-3 $\sigma$  expression by real-time RT-PCR.

The exact function and biological significance of altered 14-3-3 $\sigma$  expression in thyroid cancer cells has not thus far been elucidated. Previous studies in other tumors suggest that 14-3-3 $\sigma$  may be a prognostic marker, and high grade, advanced stage endometrial carcinomas over expressed 14-3-3 $\sigma$  when compared to low-grade lesions [13]. In these tumors, increased 14-3-3 $\sigma$  expression was correlated with hypomethylation of the CpG island. Similar findings have been reported for colorectal [19] and pancreatic cancers [35] and it has been postulated that 14-3-3 $\sigma$  over expression may be responsible for promoting proliferation and/or preventing apoptotic signal transduction [18]. Elevated 14-3-3 $\sigma$  expression has been reported as contributing to drug resistance in breast cancer cell lines [36], and its increased expression in the anaplastic cancer cell lines in the current study, may be able to explain, at least in part, the widespread resistance to chemo-therapeutic agents noted in this aggressive malignancy [37].

To summarize, we have shown that transcriptional activation of 14-3-3 $\sigma$  in thyroid cancer cells is mediated by altered methylation of its CpG island. These findings are not only confirmatory but also extend work by other investigators highlighting the epigenetic control of 14-3-3 $\sigma$  in both normal and neoplastic tissues. Additional studies are needed to further characterize the role of epigenetic mechanisms in regulating 14-3-3 $\sigma$  expression in both well-differentiated and undifferentiated primary thyroid carcinomas.

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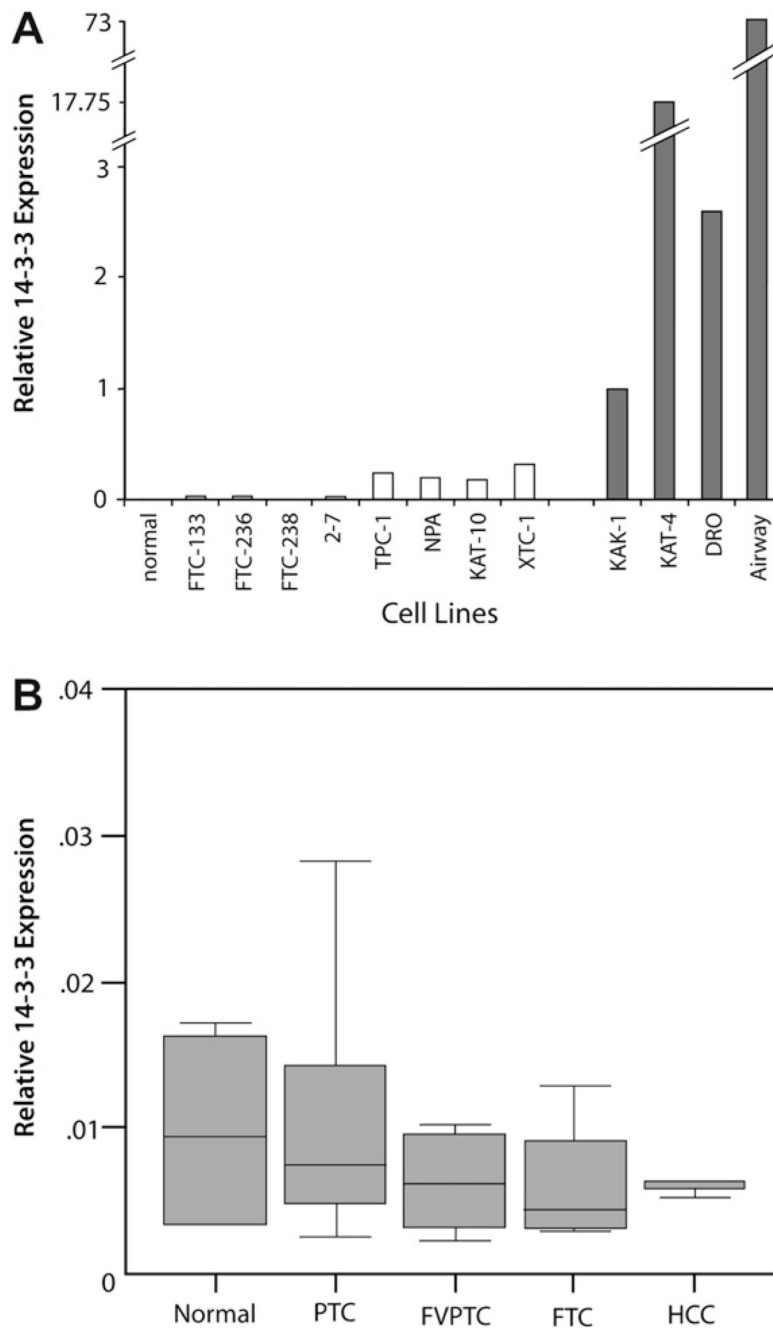
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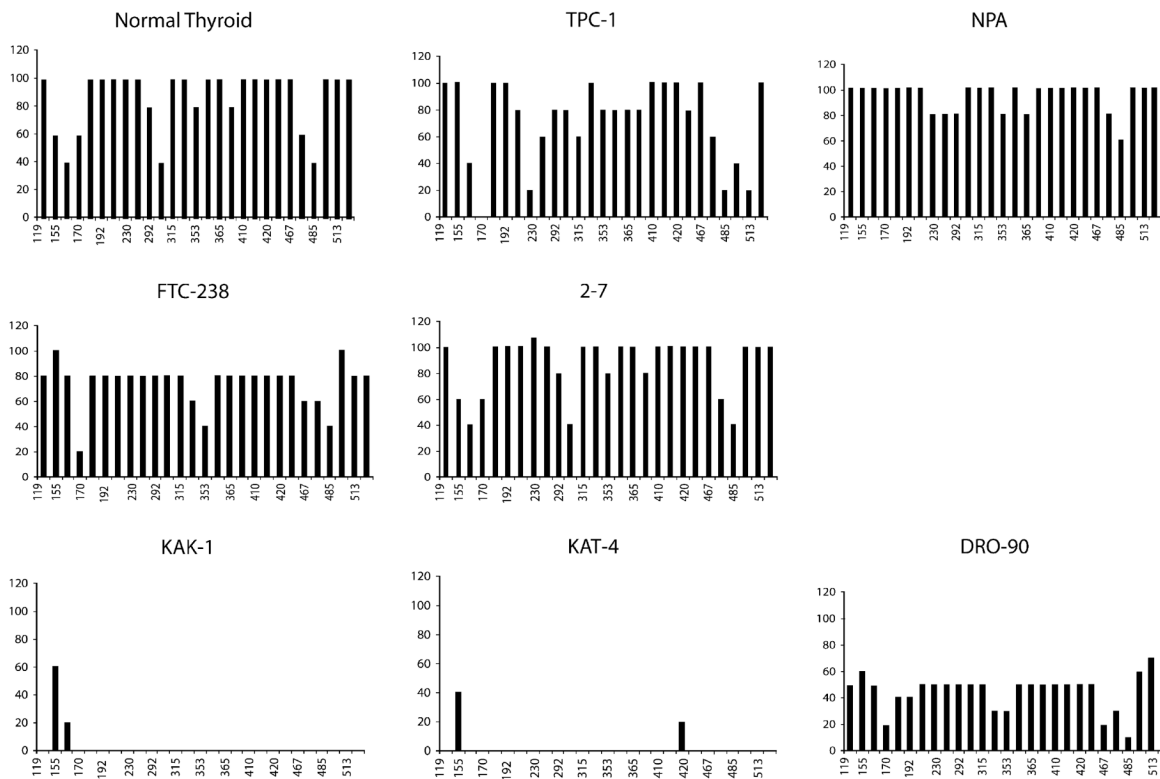


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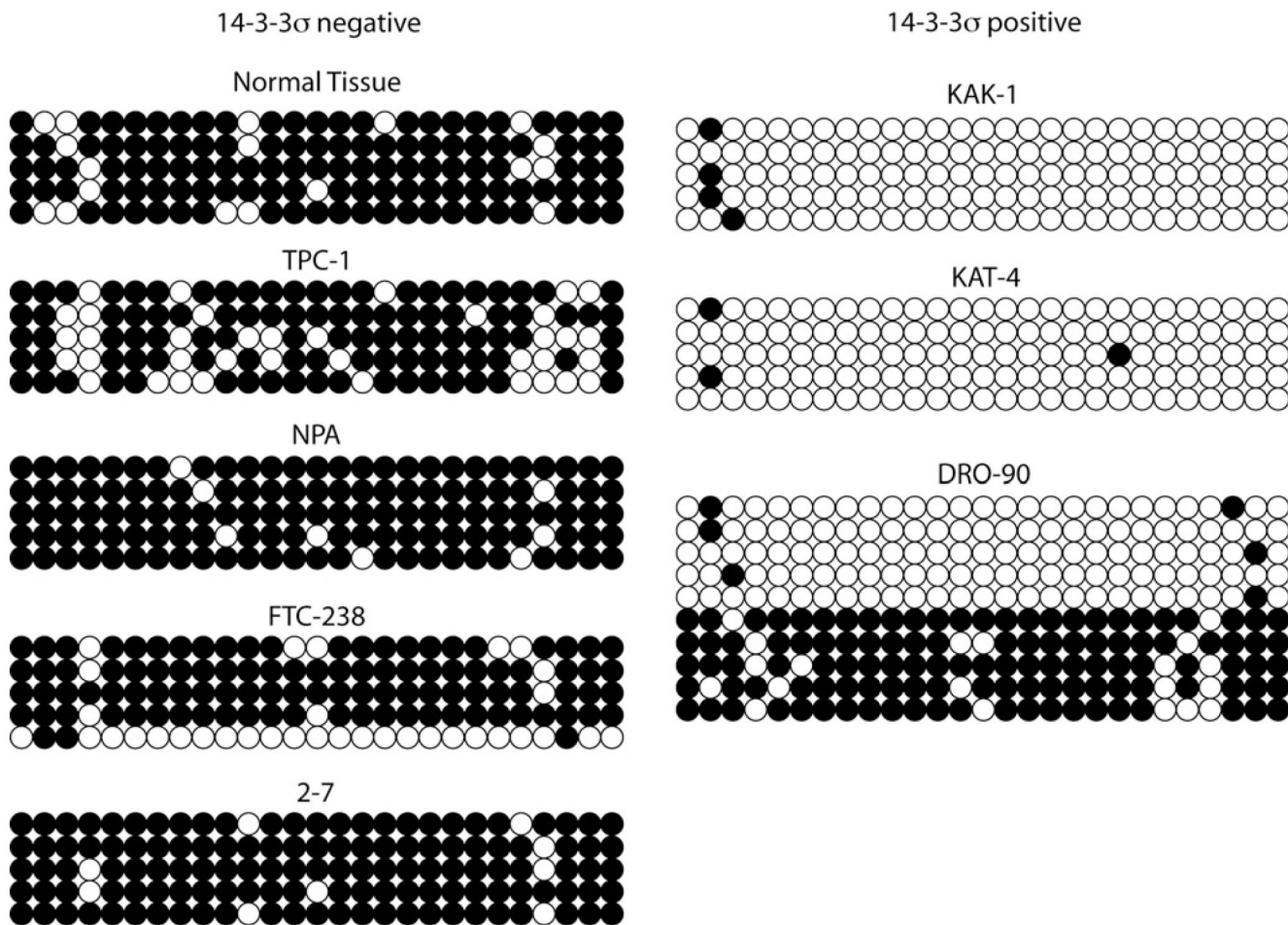


**Fig. 1.** (A) *14-3-3 $\sigma$*  expression in normal thyroid tissues (two samples) and thyroid tumor cell lines using real-time RT-PCR. The expression level in KAK-1 was designated as = 1. No expression was detected in normal thyroid tissues, all follicular (FTC) cell lines and the poorly differentiated cell lines 2-7. Very low expression levels were seen papillary tumor cell lines TPC, NPA, and KAT-10; and the Hurthle cell cancer line XTC-1. Markedly elevated *14-3-3 $\sigma$*  levels were noted in KAK-1 and the anaplastic carcinoma cell lines KAT-4 and DRO-90. Airway cells were used as a positive control for *14-3-3 $\sigma$*  expression. (B) Box plots of relative *14-3-3 $\sigma$*  expression in normal thyroid tissues and primary thyroid cancers using real-

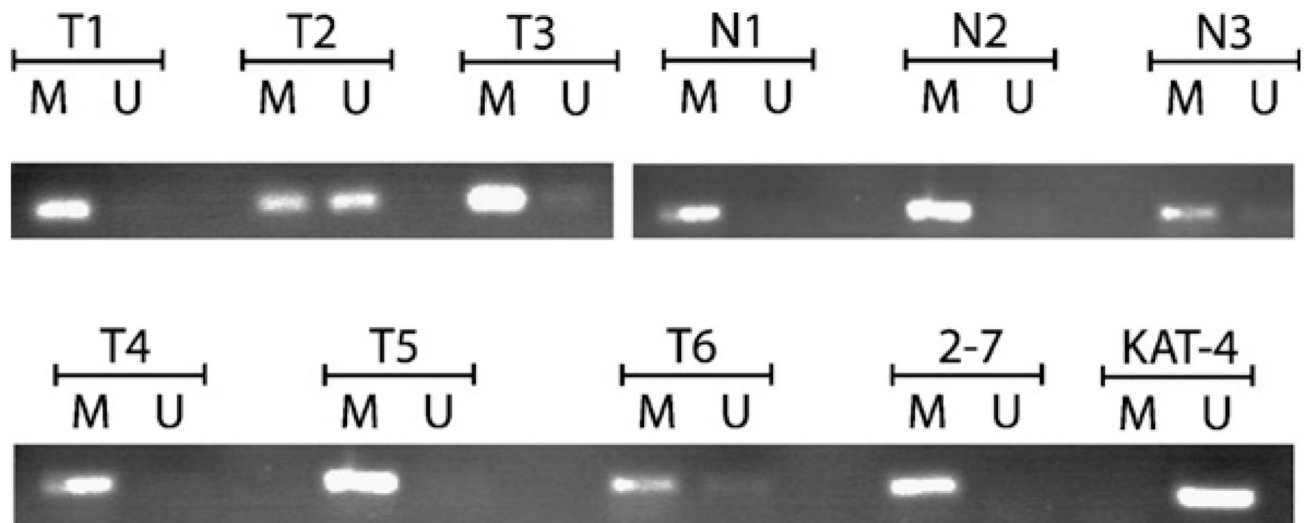
time RT-PCR. The solid lines represent the median values and the boxes represent the interquartile range (25th to 75th percentile).



**Fig. 2.** Methylation frequency at the 27 CpG sites in the *14-3-3σ* gene. The X-axis represents the nucleotide position relative to the transcription start site (as previously described) [20], and the Y-axis represents the % methylation at that site. The upper two panels represent *14-3-3σ* non-expressers, whereas the lower panel depicts the cell lines expressing *14-3-3σ* mRNA.

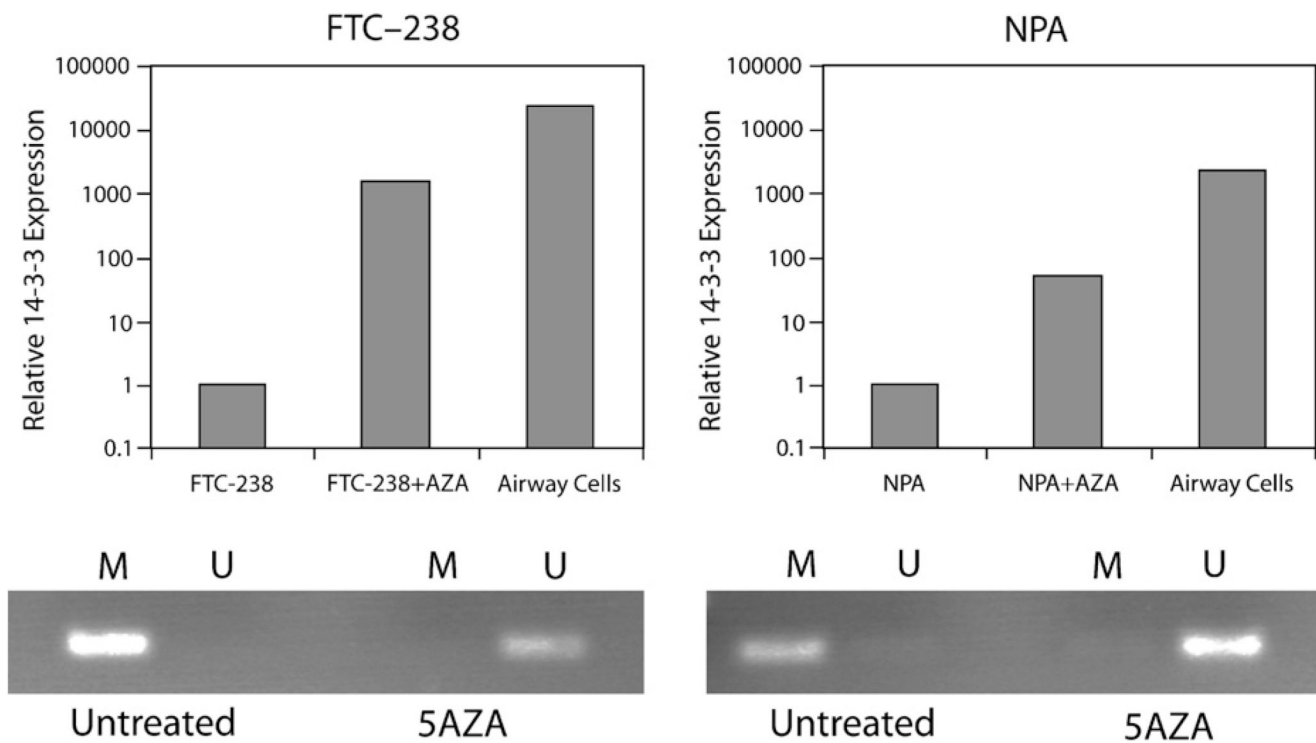


**Fig. 3.** Bisulfite sequencing of the *14-3-3σ* promoter in normal thyroid tissues and cell lines. The bubble charts show the methylation status of each of the 27 CpGs in the CpG island at the positions indicated in Fig. 2. Dark circles represent methylated cytosines, whereas open circles represent unmethylated cytosines. Each row represents a single sequenced amplicon.

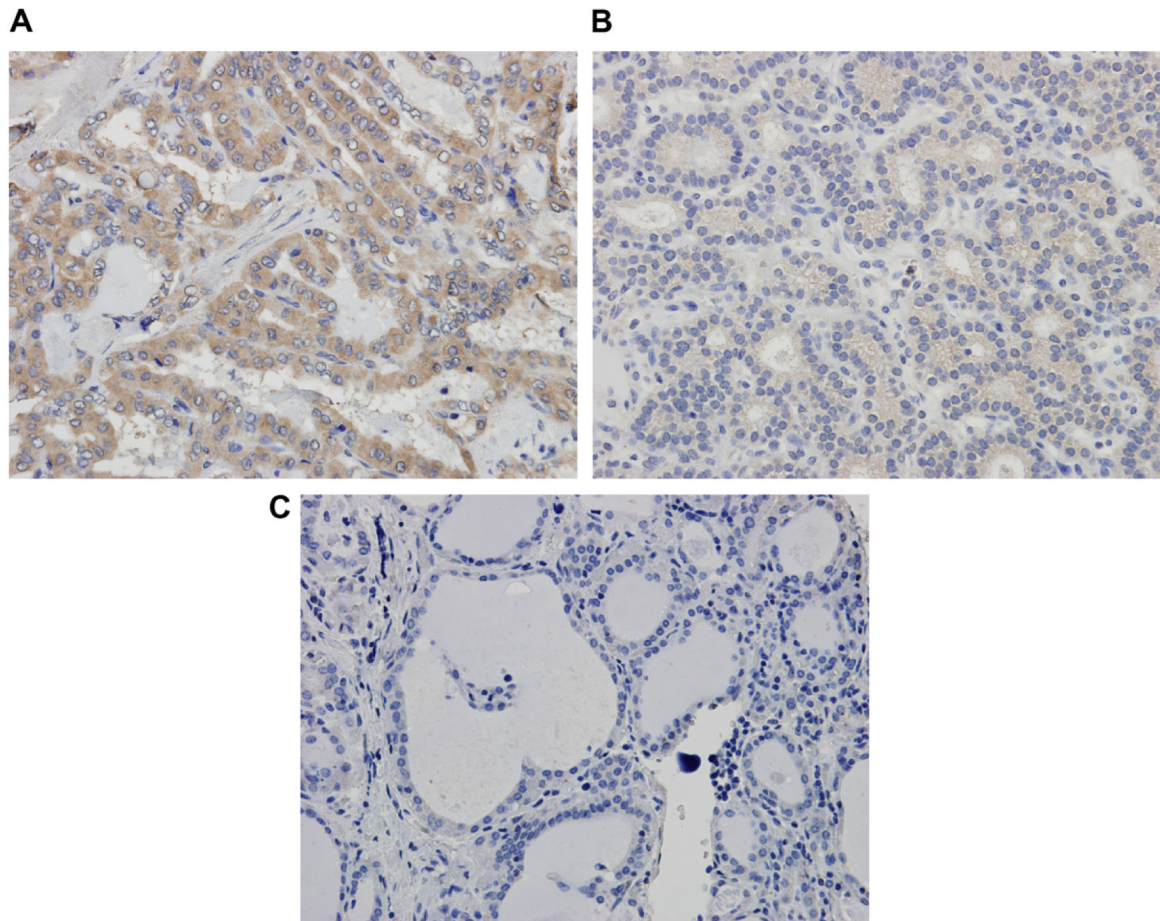


**Fig. 4.**

Methylation-specific PCR analysis of various thyroid tumor cell lines and representative primary tumors and normal thyroid tissues. Bisulfite-treated DNA was used as template for methylation-specific PCR. All the normal tissues were methylated. T1 = anaplastic cancer, T2 and T3 = papillary cancer T4 and T6 = follicular cancers, T5 = Hurthle cell cancer. Note that T2 is unmethylated. M = primers specific to methylated template DNA, U = primers specific to unmethylated template DNA, T = tumor, N = normal.



**Fig. 5.** Effect of 5-aza-dC treatment on 14-3-3 $\sigma$  mRNA expression. Treatment of FTC-238 and NPA which do not express 14-3-3 $\sigma$  at baseline, led to a marked increase in expression levels, along with demethylation of the CpG island, as shown in the lower panels. Airway cells were used as positive control for mRNA expression.



**Fig. 6.** Immunohistochemistry for 14-3-3 $\sigma$ . (A) Papillary thyroid cancer with intense staining, (B) Follicular cancer with minimal staining and (C) non-neoplastic thyroid tissue with no staining.



**Table 1**Relationship between 14-3-3 $\sigma$  expression and promoter methyl-ation frequency

Cell line	14-3-3 $\sigma$ expression	Methylation frequency (%)
Normal	-	87
KAK-1	+	3
TPC-1	-	73
NPA	-	94
KAT-10	-	n.d.
FTC-133	-	n.d.
FTC-236	-	n.d.
FTC-238	-	74
XTC-1	-	n.d.
2-7	-	93
KAT-4	+	2
DRO-90	+	34

n.d, not done.

Table 2

14-3-3 $\sigma$  IHC results by tumor type

Tissues	0	1+	2+	3+	Total
PTC	1	4	3	1	8/9 (89%)
FVPTC	2	—	1	2	3/5 (60%)
FC	2	1	—	—	1/3 (33%)
HCC	2	1	—	—	1/3 (33%)
Anaplastic	—	1	—	—	1/1 (100%)