Differential expression of two activating transcription factor 5 isoforms in papillary thyroid carcinoma

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Background: Activating transcription factor 5 (ATF5) is a member of the activating transcription/cAMP response element-binding protein family of basic leucine zipper proteins that plays an important role in cell survival, differentiation, proliferation, and apoptosis. The ATF5 gene generates two transcripts: ATF5 isoform 1 and ATF5 isoform 2. A number of studies indicate that ATF5 could be an attractive target for therapeutic intervention in several tumor types; however, so far, the role of ATF5 has not been investigated in papillary thyroid carcinoma (PTC).

Methods: Quantitative real-time reverse transcription polymerase chain reaction and immunohistochemical staining were used to study ATF5 mRNA and protein expression in PTC.

Results: We report here that ATF5 is expressed more in PTC tissue than in normal thyroid tissue. Furthermore, this is the first study that describes the presence of both ATF5 isoforms in PTC.

Conclusion: These findings could provide potential applications in PTC cancer treatment.

Keywords: papillary thyroid carcinoma, ATF5, therapeutic target, qRT-PCR, IHC

Background
Thyroid cancer is the most common type of endocrine malignancy. Four thyroid tumor types have been identified based on their histopathological features: papillary (PTC), follicular (FTC), anaplastic (ATC), and medullary thyroid carcinomas (MTC).

PTC and FTC are well-differentiated cancers. Among them, PTC is the most common histotype and accounts for more than 80%–85% of all thyroid malignant neoplasms, whereas FTC accounts for 10%–15% of cases. Another 5% is accounted for by MTC, a neuroendocrine tumor, and the remaining 1% by ATC.

Although the majority of PTC show an indolent behavior, relapses are seen in many patients. Therefore, it is important to identify new targets for PTC treatment. Several studies indicate that activating transcription factor 5 (ATF5, also known as ATF5x and ATF5γ), a transcription factor of the ATF/CREB family, regulates differentiation, cell proliferation, and survival.

Recently, we demonstrated that ATF5 plays a potential role in osteogenic differentiation. Previous studies showed that ATF5 supports cell survival by regulating the expression of antiapoptotic proteins MCL-1 in glioblastoma and BCL-2 in epithelial ovarian cancer.

Moreover, Liu et al demonstrated that Egr-1 gene was a downstream target of ATF5 that mediates ATF5-dependent cell proliferation, tumorigenic transformation, and survival.
In addition, Ishihara et al\textsuperscript{15} reported that \textit{ATF5} was one of the key molecules involved in the development of oncogenic resistance to radiotherapy, enhancing both the survival ability and malignant potential of lung cancer cells.

Therefore, \textit{ATF5} inhibition could be an attractive target for cancer therapy.\textsuperscript{15} In particular, \textit{ATF5} may be an effective method to enhance radiosensitivity in cancer cells and prevent the recurrence and progression of cancer after radiotherapy.

\textit{ATF5} is highly expressed in several cancer types, including breast, lung,\textsuperscript{16} glioma,\textsuperscript{17} B-cell chronic lymphocytic leukemia,\textsuperscript{18} rectal,\textsuperscript{19} and ovarian cancer,\textsuperscript{15} whereas it is not detectable in most normal human tissues (except the liver, where \textit{ATF5} is highly expressed).\textsuperscript{20}

Recent evidence suggests that \textit{ATF5} is overexpressed in FTC.\textsuperscript{21} In particular, Barden et al\textsuperscript{21} identified the differences in expression levels of several genes, including \textit{ATF5} in follicular thyroid adenomas and FTCs.

Despite this, however, the role of \textit{ATF5} in PTC, MTC, and ATC has not yet been investigated. In addition, the \textit{ATF5} gene generates two transcripts: \textit{ATF5} isoform 1 (\textit{ATF5}, transcript variant 1: NM_012068.5) and \textit{ATF5} isoform 2 (\textit{ATF5}, transcript variant 2: NM_001193646.1), which encodes the same single 30 kDa protein.\textsuperscript{22} These isoforms are identical in their coding regions, but differ in the 5′ untranslated region (UTR), designated as \textit{ATF5}-5′ UTR\textalpha and \textit{ATF5}-5′ UTR\textbeta. The significance of these two transcripts is presently not known and has not yet been investigated in cancer.

Recent evidence suggests that 5′ UTR\textalpha reduced the stability of \textit{ATF5} mRNA. Repression of 5′ UTR\textalpha is released by amino acid limitation or NaAsO\textsubscript{2} exposure via eIF2\textalpha phosphorylation. On the other hand, 5′ UTR\textbeta is insensitive to stress conditions.\textsuperscript{23–25}

In the present study, \textit{ATF5} mRNA and protein expression were analyzed using quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) in PTC and nonneoplastic thyroid tissue.

Moreover, we included some cases of ATC in our study. We clearly demonstrated that \textit{ATF5} was expressed more in PTC patients than in normal thyroid tissue, thus suggesting that \textit{ATF5} could represent a suitable tool to treat PTC.

\section*{Methods}

\subsection*{Samples}

Fourteen cases of PTC were retrieved from the archives of the Pathology Unit at the Mediterranean Institute of Oncology. Of these, frozen and nonneoplastic thyroid tissue was available for eleven patients. Histopathological data are summarized in Table 1.

In addition, two cases of ATC were evaluated using IHC, and one by qRT-PCR. This study was approved by the Institutional Review Board of Mediterranean Institute of Oncology, and written informed consent was obtained from all patients.

\subsection*{Total RNA extraction and reverse transcription}

Total RNA was extracted from eleven normal and PTC frozen tissues and one case of ATC using the RNeasy Mini Isolation Kit (Qiagen, Valencia, CA, USA). RNA purity was assessed by measuring the ratio of the absorbance at 260 and 280 nm.

\begin{table}[h]
\centering
\caption{Patient characteristics} \label{table1}
\begin{tabular}{|c|c|c|c|c|c|c|c|c|c|}
\hline
Age, years & Sex & Specimen type & T & N & M & Stage & Histological subtype & \textit{ATF5} IHC score in tumor & \textit{ATF5} IHC score in normal tissue \\
\hline
53 & Female & Thyroid & 1 & 0 & 0 & I & PTC & 2B & 0 \\
35 & Female & Thyroid & 1 & 0 & 0 & I & PTC & 0 & 1B \\
48 & Female & Thyroid & 1 & 0 & 0 & I & PTC & 3B & 0 \\
58 & Male & Thyroid & 1 (m) & 0 & 0 & I & PTC & 2B & 0 \\
54 & Male & Thyroid & 3 & 1 & a & 0 & III & PTC & 2B & 0 \\
27 & Male & Thyroid & 3 & 1 & 0 & I & PTC & 3B & 0 \\
52 & Female & Thyroid & 1 & 0 & 0 & I & PTC & 4C & 3B \\
80 & Female & Thyroid & 3 & 0 & 0 & III & PTC & 2B & 2A \\
40 & Female & Thyroid & 3 & 0 & 0 & III & PTC & 3B & 2C \\
74 & Female & Thyroid & 3 & 0 & 0 & III & PTC & 2B & 2B \\
31 & Male & Thyroid & 1 & 1 & 0 & I & PTC & 2B & 1B \\
61 & Female & Thyroid & 1 & 0 & 0 & I & PTC & 4B & 4A \\
33 & Female & Thyroid & 3 & 1 & 0 & I & PTC & 2B & 2B \\
\hline
\end{tabular}
\end{table}

\textbf{Notes:} The table includes details of 14 cases of papillary thyroid carcinoma in different stages; there were 4 males and 10 females. Age range of patients was 27–80 years, with a mean age of 50 years.

\textbf{Abbreviations:} \textit{ATF5}, activating transcription factor 5; IHC, immunohistochemistry; PTC, papillary thyroid carcinoma; T, tumor; N, lymph node; M, metastasis; m, multifocal.
280 nm, considering 1.8–2 as admissible range of ratios for pure RNAs. RNA quality was analyzed using Agilent 2100 Bioanalyzer RNA assays (Agilent Technologies, Santa Clara, CA, USA) and evaluated by calculating the ratio of the 28S and 18S ribosomal RNA intensity peaks. Total RNA was stored at −80°C.

RNA samples (1 μg) were reverse transcribed using the High-Capacity cDNA Reverse Transcription (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Reverse transcription products were stored at −20°C.

**Real-time RT-PCR**

qRT-PCR was performed using EUR™ SG qPCR Master Mix according to the manufacturer’s protocol on a HT7900 instrument (Applied Biosystems). Primers were designed using Primer BLAST™ and specifically recognize selected mRNAs by targeting exon–exon junctions (Table 2). ATF5 transcriptional isoforms are differentially amplified according to dissimilarities in 5′ UTRs. Specificity of the amplification products was confirmed by examination of dissociation reaction plots. A distinct single peak indicated that a single DNA sequence was amplified during PCR. Each sample was tested in triplicate, and samples were used for analysis of relative gene expression using the 2−ΔΔCT method. Reported ΔCT values are inversely proportional to target mRNA concentration.

**Immunohistochemistry**

ATF5 expression was analyzed using IHC on 14 PTC formalin-fixed paraffin-embedded specimens and adjacent normal thyroid tissues and two ATCs. Paraffin blocks were cut as 3-μm sections. Tissue sections were dewaxed with xylene and rehydrated in descending concentrations of ethanol (100% and then 75%). Antigen retrieval was performed with a pH 8.0 EDTA solution at 95°C for 40 minutes in a Decloaking Chamber (Biocare Medical, Walnut Creek, CA, USA). After rinsing briefly in phosphate-buffered saline (PBS 1×), sections were incubated for 10 minutes with 3% H2O2 at room temperature in the dark and washed again.

Afterward, the sections were permeabilized with Triton 0.4% for 30 minutes. Subsequently, the nonspecific binding of the antibodies was blocked with 4% blocking serum (bovine serum albumin; Sigma Aldrich Co., St Louis, MO, USA) for 30 minutes. The sections were next stained with a rabbit anti-ATF5 antiserum (Novus Biologicals, Littleton, CO, USA) at a ratio of 1:500 in a humidified chamber overnight at 4°C.

The following day, sections were washed three times (5 minutes each) in PBS 1×.

Visualization was achieved by exposing the sections to Envision + Dual Link System-HRP (Dako Denmark A/S, Glostrup, Denmark) for 30 minutes at room temperature. The sections were then incubated with dianmonobenzidine solution and rinsed with PBS 1×. The slides were counterstained with Harris’s hematoxylin for 1 minute at room temperature and, after dehydration, sealed with a drop of mounting medium.

Interpretation of staining was done using a semiquantitative system. The percentage of stained cells was assessed as follows: 1=1%–25%; 2=26%–50%; 3=>51%–75%; 4=>76%–100%, whereas staining intensity was scored as follows: A = low; B = mid; C = strong (Table 1).

### Statistical analyses

Differences in ATF5 transcripts expression between normal and tumor samples were assessed using paired Student’s t-test. ATF5 staining has been evaluated comparing both the percentage of ATF5-positive cells and the intensity of the signal in tumor tissues and adjacent area using Fisher’s exact test. Comparisons resulting in P-values <0.05 were reported as statistically significant. Statistical calculation was performed using the software R (version 3.2.1, Lucent Technologies, Murray Hill, NJ, USA).27

### Table 2 List of primers used in real-time PCR analysis

<table>
<thead>
<tr>
<th>Target transcript</th>
<th>GenBank ID</th>
<th>Primer sequence</th>
<th>Temp Fwd/Rev (°C)</th>
<th>Prod size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATF5 transcript variant 1</td>
<td>NM_012068.5</td>
<td>Fwd: CAGGAATTTCTGTCAAAGCAGGAA&lt;br&gt;Rev: CCGGCACTCTCTCCTCCTG</td>
<td>60/62</td>
<td>220</td>
</tr>
<tr>
<td>ATF5 transcript variant 2</td>
<td>NM_001193646.1</td>
<td>Fwd: TGCTCCTGAGTACAGTCTCT&lt;br&gt;Rev: AAGTGGAGACTCTCTGGCCTG</td>
<td>60/60</td>
<td>201</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046.5</td>
<td>Fwd: GCCATTCCAGACATCCAACCTGCC&lt;br&gt;Rev: GGCTTGCTATACACAGGAATAGCTT</td>
<td>65/63</td>
<td>347</td>
</tr>
<tr>
<td>TUBB</td>
<td>NM_178014.3</td>
<td>Fwd: CCCAGAACATTTGGCAGCAT&lt;br&gt;Rev: GCGATGCTACAGTCAACATGCC</td>
<td>59/60</td>
<td>166</td>
</tr>
</tbody>
</table>

**Abbreviations:** ATF5, activating transcription factor 5; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PCR, polymerase chain reaction; TUBB, tubulin beta chain; Prod, product; bp, base pair; Fwd, forward; Rev, reverse.
Results

ATF5 expression in PTC

mRNA levels of ATF5 isoforms 1 and 2 were examined by real-time PCR analysis on eleven PTC and adjacent normal samples using the primers listed in Table 2.

Our results indicate that ATF5 isoform 1 mRNA level was significantly higher ($P=0.0477$) in PTC when compared to adjacent normal tissue ($\Delta\text{CT}$ medians: 3.3 for tumor and 3.9 for normal); ATF5 isoform 2 was not statistically significant ($\Delta\text{CT}$ medians: 3.3 for tumor and 3.5 for normal). Also, there was a statistically significant difference ($P=0.046$) when both isoforms were considered together ($\Delta\text{CT}$ medians: 6.6 for tumor and 7.3 for normal). These data are shown in Figure 1.

Such findings suggest that ATF5 is expressed more in PTC patients than in normal samples, and hence this could be an attractive therapeutic target for PTC cancer treatment.

ATF5 expression in ATC

mRNA levels of ATF5 isoforms 1 and 2 were also examined by real-time PCR analysis on one ATC and adjacent normal samples. The results showed an increased expression of both isoforms in ATC patients than in normal sample (data not shown).

IHC ATF5 expression

To confirm these data, we performed an anti- ATF5 immunohistochemical analysis on 14 PTC specimens. We found higher ATF5 expression in PTC when compared to adjacent normal thyroid tissue (Figure 2), confirming the data obtained from real-time PCR analysis.

Representative staining of ATF5 protein expression in normal and PTC tissues is shown in Figure 3. Afterward, we performed an anti-ATF5 immunohistochemical analysis on two ATC specimens. Interestingly, we observed a strong expression of the protein (data not shown).

Discussion

ATF5 is a stress response transcription factor whose expression is regulated by posttranscriptional, translational, and posttranslational regulation in response to cellular stresses. Although studies have reported that ATF5 is involved in the progression of several tumor types, its expression in PTC has never been studied. Our results are in line with previous data,
which reports that in general, the expression level of \textit{ATF5} is significantly higher in malignant tissues than their normal counterparts. The only exception appears to be hepatocellular carcinoma cells, which express lower levels of \textit{ATF5} than normal liver cells.\textsuperscript{20}

Furthermore, several studies have reported that high \textit{ATF5} expression levels may correlate with poor prognosis in cancer patients.\textsuperscript{12} In our study, however, there was no significant association between \textit{ATF5} expression and size of the tumor, local extension, lymph node involvement, or distant metastasis.

Interestingly, we observed a strong expression of the protein in some ATC included in our study. ATC is an undifferentiated carcinoma characterized by an aggressive behavior and a poor prognosis. Further analysis with real-time PCR confirmed an increased expression of both isoforms.

Moreover, more data on ATC would be necessary to confirm our data in a larger cohort of patients.

The main biological function of \textit{ATF5} is related to apoptosis, and several studies have been conducted so far to investigate the role of \textit{ATF5} as a possible therapeutic target. Interference with \textit{ATF5} function or expression in glioma cells and breast cancer cell lines caused marked apoptotic cell death.\textsuperscript{16,28} Moreover, loss of function of \textit{ATF5} in conjunction with paclitaxel treatment elicited apoptosis of pancreatic carcinoma cells.\textsuperscript{29}

In addition, Karpel-Massler et al.\textsuperscript{30} demonstrated that a dominant-negative \textit{ATF5} peptide induces apoptosis in glioblastoma, triple-negative breast cancer (MDA-MB-436),

**Figure 2** Increased \textit{ATF5} expression in immunohistochemical analyses performed on PTC patients.

\textbf{Notes:} Immunohistochemical analysis of \textit{ATF5} expression in 14 formalin-fixed paraffin-embedded specimens from PTC patients and adjacent normal tissue. Differential immunohistochemical expression of \textit{ATF5} in normal and tumor tissue. (A) indicates the percentage of positive cells, while in (B), the staining intensity.

\textbf{Abbreviations:} \textit{ATF5}, activating transcription factor 5; PTC, papillary thyroid carcinoma.

**Figure 3** Immunohistochemical expression of \textit{ATF5} in PTC.

\textbf{Notes:} Immunohistochemical expression of \textit{ATF5} in papillary thyroid carcinoma (A), 20×, and adjacent normal thyroid tissue (B), 20×.

\textbf{Abbreviations:} \textit{ATF5}, activating transcription factor 5; PTC, papillary thyroid carcinoma.
hormone-refractory prostate cancer (PC3 and DU145), resis-
tant non-small-cell lung cancer (H1975), BRAF (V600E)-
mutated melanoma (A375), and pancreatic carcinoma
(PANC-1). This dominant-negative ATF5 also showed
in vivo efficacy in reducing the growth of a range of tumor
types in xenograft models.

To our knowledge, this study provides the first evaluation
of ATF5 expression and identification of its two isoforms in
PTC and normal tissue. Targeting of ATF5 could potentially
become a viable therapeutic approach for patients diagnosed
with PTC.

Conclusion

In conclusion, our data revealed that ATF5 mRNA expression
and protein was higher in PTC compared to normal thyroid
tissue. Our results unambiguously demonstrate the existence
of both ATF5 isoforms in PTC.

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assistance.

Author contributions

LV designed the study, analyzed the results, and wrote the manu-
script. CLR performed IHC and contributed to data analysis. SF
and GC performed real-time polymerase chain reaction experi-
ments and contributed to data analysis. SS provided the surgical
samples and clinical data. EA performed IHC. LM and CC
analyzed IHC and critically revised the manuscript. All authors
contributed toward data analysis, drafting and revising the paper
and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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