

**FOXA1 Is a Potential Oncogene in Anaplastic Thyroid Carcinoma**

Carmelo Nucera,1 Jerome Eeckhoute,1 Stephen Finn,2 Jason S. Carroll,3 Azra H. Ligon,2,10 Carmen Priolo,4 Guido Fadda,5 Mary Toner,6 Orla Sheils,7 Marco Attard,8 Alfredo Pontecorvi,9 Vania Nose,10 Massimo Loda,2,4,10 and Myles Brown1,11

**Abstract**

**Purpose:** FOXA1 is a mammalian endodermal transcription factor belonging to the human forkhead box gene family that plays a role in certain tumor types. Here, we investigated the potential role of FOXA1 in human thyroid carcinomas.

**Experimental Design:** We examined the level of FOXA1 expression and gene copy number by immunohistochemistry and fluorescence in situ hybridization, respectively, in a cohort of benign and malignant thyroid tumors. In addition, we examined the role of FOXA1 in the proliferation of an undifferentiated thyroid carcinoma cell line by short hairpin RNA-mediated silencing.

**Results:** We show that FOXA1 is overexpressed in human anaplastic thyroid carcinomas (ATC). In addition, we identify FOXA1 DNA copy number gain within the 14q21.1 locus in both an ATC cell line and human ATC cases. Silencing of FOXA1 in an ATC cell line causes G1 growth arrest and reduction of cell proliferation. Moreover, we observe a potential link between FOXA1 and the cell cycle machinery by identifying p27kip1 up-regulation on FOXA1 silencing.

**Conclusions:** FOXA1 is overexpressed in aggressive thyroid cancers and involved in cell cycle progression in an ATC cell line. Therefore, FOXA1 may be an important oncogene in thyroid tumorigenesis and a potential new therapeutic target for the treatment of anaplastic thyroid carcinomas.

Malignant tumors of thyroid follicular cells have been classified as either well-differentiated thyroid carcinomas, represented by papillary and follicular thyroid carcinomas, or undifferentiated (anaplastic) thyroid carcinoma (ATC). Although the majority of either well-differentiated thyroid carcinomas are indolent malignancies with a 10-year survival of up to 90% (1), ATC, which represents ~2% of all thyroid cancers, is one of the most aggressive malignancies known, rapidly invading the neck and often spreading to other organs (2). ATC is thought to develop from an existing papillary or follicular cancer and is responsible for more than half of thyroid carcinoma deaths, with a mean survival of 6 months from diagnosis (3). In fact, neither chemotherapy nor radiation therapy are effective to prolong ATC patient survival (4). Therefore, alternative systemic treatment for this cancer are urgently needed.

ATC have a very high proliferation rate and marked aneuploidy (5). Several genes involved in cell proliferation and cell cycle regulation have been shown to be dysregulated in these tumors (6). In addition, it has been recently reported that thyroid-specific transcription factors crucial for thyroid organogenesis and differentiation, such as Pax8, TTF-1, and TTF-2 (FOXE1), are expressed in thyroid neoplasms (7). In particular, Pax8 is highly expressed in ATC, whereas TTF-1 and FOXE1 were seen in a lower percentage (7).

FOXA factors are members of the winged helix/forkhead box family and play important roles during multiple phases of mammalian life, regulating development of endodermal primordia and organogenesis as well as regulating metabolism and homeostasis in the adult (8–10). In particular, FOXA1 (hepatocyte nuclear factor 3α) is involved in hepatic differentiation and is an endodermal “pioneer transcription factor,” binding to promoters and enhancers enabling chromatin access for other tissue-specific transcription factors (11, 12). This transcription
Western blot (Seven Hills Bioreagents), p27kip1 (Transduction Laboratories), FOXA1 (Ab5089 and Ab23738) for Western blot. The experiments were done at least three times in triplicate to ensure reproducibility of the results.

Antibodies

The following antibodies were used: β-actin (Sigma), lamin A/C (Cell Signaling Technology), FOXA1 (Ab5089 and Ab23738) for Western blot (Abcam), FOXA1 clone 2F83 for immunohistochemistry and Western blot (Seven Hills Bioreagents), p27kip1 (Transduction Laboratories), cleaved poly(ADP-ribose) polymerase (Cell Signaling Technology), cyclin D1 (Thermo Scientific), MIB-1 (DAKO), p21 (BD Pharmingen), and cyclin E1 (Santa Cruz Biotechnology).

Human thyroid cell lines

The human thyroid carcinoma cell lines used in the study were BCPAP and TPC-1 (papillary thyroid carcinoma cell lines), WRO82-1 (follicular thyroid carcinoma cell line), and 8505c (undifferentiated thyroid carcinoma cell line). BCPAP were kindly provided by Dr. G. Damante (University of Udine) and WRO cell line was established by Dr. G.F. Juilland (University of California at Los Angeles) and provided by Dr. F. Frasca (University of Catania). TPC-1 cell line was kindly provided by Dr. F. Frasca. 8505c cell line (positive for cytokeratins, markers of epithelial tumors) was established by Dr. M. Akiyama (Radiation Effects Research Foundation). TE4 cell line (esophageal squamous adenocarcinoma) was provided by Dr. H. Ishii (Center for Molecular Medicine, Ichi Medical School). MCF-7 and MDA-231 (human breast cancer cell lines) were grown as in Eekhoute et al. (19). All cell lines were grown in RPMI (BCPAP cells were grown in DMEM) supplemented with 10% FCS and ampicillin/streptomycin.

Cell transfections

For transfection assays, cells were grown in 60 mm well plates and transfected using Fugene-6 (Roche) in Opti-MEM for 48 h according to the manufacturer’s instructions. Experiments were done at least three times in triplicate to ensure reproducibility of the results.

Short hairpin RNA

To stably suppress FOXA1, we used pLKO.1 lentiviral constructs containing two short hairpin RNA (shRNA) sequences generated by the RNAi Consortium, sh#1 anti-FOXA1 GCAGTACTACCAAGGGTTGT-TAT and sh#2 anti-FOXA1 GCAGCATAAGCTGGACCTCA. 8505c cells were also infected with the shRNA anti-GFP control. Lentiviral infections were done as follows. Briefly, 0.5 × 10^6 293T cells were seeded in 60 mm dishes and the day after cotransfected with pLKO.1 lentiviral constructs and packaging plasmids (gag-pol and VSV-G) using Fugene 6 (Roche). The medium containing the progeny virus released from the 293T cells was filtered by 0.45 μm filters, collected, and used to infect the 8505c cells for 3 to 6 h in the presence of 8 μg/mL polybrene (Sigma). The 8505c cells were incubated for additional 48 h, selected with puromycin (Sigma-Aldrich) for 48 h, and then lysed for Western blotting and real-time PCR analyses. FOXA1 knockdown was confirmed by quantitative real-time PCR and Western blot. The experiments were done at least three times in triplicate to ensure reproducibility of the results.

Real-time PCR

RNA isolation and real-time reverse transcription-PCR were done as reported from Keeton and Brown (20). We used the following primers: GAAGATGGAGGCCATGAAA and GCTCTAGTTCATGTTTGCA for FOXA1, AAATAAGGACACCCCTGGA and ATITGGGGAACCGTCTGAA for p27kip1, CCAAAAGTCAAGTCTCTTGTTG and CATGAGTGTC- GAGCACATC for p21waf1, TGAGGCTTCGAGAACACAGAA and TGGACGGGCCTTTTTTCA for cyclin D1, and TACCCCAAATC- CAAGGTGCAC and CATGATTTTCGCCATTTTC for cyclin E1. FOXA1 mRNA expression was normalized to the 40 ribosomal protein S28. The presence of a single amplicon was systemically verified by disassociation curve analysis.

Nuclear and cytoplasmic fractionation

We used a lysis buffer composed of 1 mol/L HEPES, 5 mol/L NaCl, 1 mol/L MgCl_2, glycerol, Triton X-100, and 1 mol/L DTT. We scraped cells and washed twice in cold PBS, added 300 μL lysis buffer, and left the cells on ice 5 min, centrifuged the lysate at 2,800 rpm for 1 min at 4°C, and collected the supernatant (cytoplasmic fraction). We resuspended the pellet in 200 μL lysis buffer, added 28 μL of 5 mol/L NaCl, and mixed for 1 h at 4°C on a nutator, centrifuged the mixture at 14,000 rpm for 10 min at 4°C, and collected the supernatant (nuclear fraction).

Immunoblot analysis

Western blot assays were done as in Keeton and Brown (20). Bands were quantified by densitometry with the AlphaEase software (Alpha Innotech) according to the manufacturer’s instructions.

Cell cycle analysis and bromodeoxyuridine assay

sh-control, sh#1-FOXA1, and sh#2-FOXA1 8505c cells were seeded at 2 × 10^3 per well in a six-well plate and grown. After 48 h, bromodeoxyuridine (BrdUrd; Upstate Cell Signaling Solutions) was added to a final concentration of 10 μmol/L for 1 h. 8505c cells were trypsinized and fixed in ethanol 75% prechilled (~20°C). The pelleted cells at 400 × g at room temperature were resuspended in PBS/0.5% bovine serum albumin (BSA), denatured in 2 mol/L HCl/0.5% BSA for 30 min, and then neutralized with 3 mol/L NaOH/30 mmol/L Tris-HCl. The DNA pellet was washed, resuspended in PBS, and incubated for 30 min at 37°C before analysis by flow cytometry.

Translational Relevance

Anaplastic thyroid carcinoma (ATC) is highly aggressive and is responsible for more than half of thyroid carcinoma deaths, with a median survival of 3 to 6 months from diagnosis. Neither chemotherapy nor radiation therapy are effective in prolonging ATC patient survival; therefore, alternative systemic treatments for this cancer are urgently needed. FOXA1 is a "pioneer transcription factor" overexpressed in some human cancers and involved in estrogen-dependent growth in breast cancer. We found FOXA1 overexpression and gene copy number gain in >70% of ATC. In addition, we show that silencing of FOXA1 leads to the growth arrest of ATC cell lines by reversing its inhibition of p27kip1 transcription. These results suggest that FOXA1 is a potential ATC oncogene and that therapies leading to the down-regulation of FOXA1 could represent a novel therapeutic strategy. Development of FOXA1-directed therapies would be targeted to those tumors harboring FOXA1 copy number gains at 14q21.1.

Materials and Methods

Human thyroid cell lines

The human thyroid carcinoma cell lines used in the study were BCPAP and TPC-1 (papillary thyroid carcinoma cell lines), WRO82-1 (follicular thyroid carcinoma cell line), and 8505c (undifferentiated thyroid carcinoma cell line). BCPAP were kindly provided by Dr. G. Damante (University of Udine) and WRO cell line was established by Dr. G.F. Juilland (University of California at Los Angeles) and provided by Dr. F. Frasca (University of Catania). TPC-1 cell line was kindly provided by Dr. F. Frasca. 8505c cell line (positive for cytokeratins, markers of epithelial tumors) was established by Dr. M. Akiyama (Radiation Effects Research Foundation). TE4 cell line (esophageal squamous adenocarcinoma) was provided by Dr. H. Ishii (Center for Molecular Medicine, Ichi Medical School). MCF-7 and MDA-231 (human breast cancer cell lines) were grown as in Eekhoute et al. (19). All cell lines were grown in RPMI (BCPAP cells were grown in DMEM) supplemented with 10% FCS and ampicillin/streptomycin.
20 min at room temperature, washed with PBS/0.5% BSA, and centrifuged 5 min at 400 × g at room temperature. The cells were resuspended in 0.1 mol/L sodium borate (pH 8.5) for 2 min at room temperature, washed with PBS/0.5% BSA, centrifuged 5 min at 400 × g at room temperature, and resuspended in a solution PBS 0.5% Tween 20 containing FITC-conjugated anti-BrdUrd monoclonal antibody (BD Biosciences) according to the manufacturer’s instructions and incubated in the dark for 30 min at 37°C. Finally, the cells were pelleted, washed twice with PBS/0.5% BSA, and resuspended in 500 μL of this solution. Propidium iodide was added to a final concentration of 10 μg/mL with RNase (10 mg/mL). Cells were incubated at room temperature for 30 min and then analyzed by flow cytometry on a FACSCalibur (Becton Dickinson Immunocytometry Systems) exciting at 488 nm and measuring the BrdUrd-FITC on the green fluorescence through a 514 nm bandpass filter and the DNA-linked red fluorescence (propidium iodide) through a 600 nm low pass filter, with compensation to reduce overlap between red and green fluorescence.

**Apoptosis assay**

Following 48 h sh-control, sh#1-FOXA1, and sh#2-FOXA1 8505c cells were collected and fixed overnight at 4°C with 75% ethanol for propidium iodide staining and flow cytometry analysis on a FACSCalibur to evaluate sub-G1 cell populations. Proteins were extracted at the same time point and assayed for cleaved poly(ADP-ribose) polymerase expression by Western blot.
Pellets were then processed in a tissue processor in the usual way. (Richard-Allan Scientific) were added to the cell pellet dropwise. The for 15 min. After aspiration of the ethanol, 2 mL of liquefied Histogel ing afurther PBS wash, the cells were then resuspended in 70% ethanol resuspended in 4% paraformaldehyde for 15 min to fix them. Follow- down (1,200 rpm for 5 min). The PBS was aspirated and the cells were harvested in PBS, human cancer cell lines were gently spun Cell pellet processing

An institutional review board protocol was approved by V.N. for the ATC cases were screened for TTF-1, thyroglobulin, calcitonin, carcinoembryonic antigen, and cytokeratins. All the carcinomas displaying extensive immunoreactivity for calcitonin and/or carcinoembryonic antigen, and cytokeratins. All the carcinomas displaying

Table 1. FOXA1 nuclear expression in human primary ATC

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Gender</th>
<th>Histopathology</th>
<th>FOXA1 localization</th>
<th>Positive nuclei*</th>
<th>FISH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64</td>
<td>M</td>
<td>ATC squamous</td>
<td>Nuclear</td>
<td>4</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>F</td>
<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>4</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>F</td>
<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>2</td>
<td>NR†</td>
</tr>
<tr>
<td>4</td>
<td>65</td>
<td>M</td>
<td>ATC osteoclastic like giant cells</td>
<td>Nuclear</td>
<td>2</td>
<td>NR</td>
</tr>
<tr>
<td>5</td>
<td>93</td>
<td>F</td>
<td>ATC spindle and giant cells</td>
<td>Nuclear</td>
<td>4</td>
<td>Disomy</td>
</tr>
<tr>
<td>6</td>
<td>80</td>
<td>M</td>
<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>3</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>7</td>
<td>68</td>
<td>M</td>
<td>ATC spindle and giant cells</td>
<td>Nuclear</td>
<td>4</td>
<td>Disomy</td>
</tr>
<tr>
<td>8</td>
<td>81</td>
<td>F</td>
<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>4</td>
<td>Polysomy plus low-level gain of FOXA1</td>
</tr>
<tr>
<td>9</td>
<td>54</td>
<td>F</td>
<td>ATC + extensive PDTC + PTC foci</td>
<td>Nuclear</td>
<td>3</td>
<td>NR</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>F</td>
<td>ATC squaromait features</td>
<td>Nuclear</td>
<td>3</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>11</td>
<td>68</td>
<td>M</td>
<td>ATC + PTC follicular variant foci</td>
<td>Nuclear</td>
<td>3</td>
<td>Polysomy plus low-level gain of FOXA1</td>
</tr>
<tr>
<td>12</td>
<td>52</td>
<td>M</td>
<td>ATC giant cells, rhabdoid differentiation + rhabdomysosarcomatomous transformation</td>
<td>Nuclear</td>
<td>3</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>13</td>
<td>70</td>
<td>F</td>
<td>ATC sarcomatoid variant, giant cells + PTC foci</td>
<td>Nuclear</td>
<td>4</td>
<td>Monosomy</td>
</tr>
<tr>
<td>14</td>
<td>77</td>
<td>M</td>
<td>ATC + focal PDTC + PTC foci</td>
<td>Nuclear</td>
<td>1</td>
<td>Gain/polysomy</td>
</tr>
<tr>
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<td>43</td>
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<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>4</td>
<td>NR</td>
</tr>
<tr>
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<td>43</td>
<td>M</td>
<td>ATC spindle cells</td>
<td>Nuclear</td>
<td>4</td>
<td>NR</td>
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<tr>
<td>17</td>
<td>74</td>
<td>F</td>
<td>ATC spindle cells, plasmacytoid and rhabdoid features</td>
<td>Nuclear</td>
<td>1</td>
<td>Monosomy</td>
</tr>
<tr>
<td>18</td>
<td>76</td>
<td>F</td>
<td>ATC osteoclastic like, giant cells + oncocytic PTC foci</td>
<td>Nuclear</td>
<td>2</td>
<td>Gain/polysomy</td>
</tr>
<tr>
<td>19</td>
<td>77</td>
<td>F</td>
<td>ATC + PTC foci</td>
<td>No</td>
<td>1</td>
<td>NR</td>
</tr>
<tr>
<td>20</td>
<td>87</td>
<td>F</td>
<td>ATC + PTC foci</td>
<td>Nuclear</td>
<td>2</td>
<td>NR</td>
</tr>
</tbody>
</table>

Abbreviations: PDTC, poorly differentiated thyroid carcinoma; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma.
*Positive nuclei: 1, negative; 2, 1% to 10% of examined nuclei positive (low expression); 3, 11% to 50% of nuclei positive (high expression); and 4, >50% of nuclei positive (high expression).
† No results.

Paraffin-embedded thyroid tumors and tissue microarrays

Thyroid tumors were classified according to WHO diagnostic criteria (21). Tissue microarrays were composed from 177 cases: 15 normal thyroid tissues, 30 follicular adenomas, 58 nodular hyperplasia samples, 8 lymphocytic thyroiditis samples, 6 Graves, and 60 differentiated thyroid carcinomas: 48 papillary thyroid carcinomas (31 classic, 13 follicular variant, 3 microcarcinomas, and 1 oxyphilic variant), and 12 follicular thyroid carcinoma. Tissue microarrays were constructed according to previously established procedure (22) from anonymized archival blocks of formalin-fixed, paraffin-embedded tissues from the files of the Central Pathology Laboratory at St. James’s Hospital, Dublin.

We studied 3 insular thyroid carcinomas and 20 ATC from 20 patients who underwent thyroidectomy (9 males and 11 females; average age, 67.3 ± 13.9 years; range, 43-93 years).

The ATC cases were screened for TTF-1, thyroglobulin, calcitonin, carcinomaembryonic antigen, and cytokeratins. All the carcinomas displaying extensive immunoreactivity for calcitonin and/or carcinomaembryonic antigen (medullary carcinoma) were not included in the series. Finally, all cases were reviewed by four pathologists (G.F., M.L., O.S., and V.N.).

An institutional review board protocol was approved by V.N. for the acquisition of paraffin-embedded thyroid tissue.

Cell pellet processing

After harvesting in PBS, human cancer cell lines were gently spun down (1,200 rpm for 5 min). The PBS was aspirated and the cells were resuspended in 4% paraformaldehyde for 15 min to fix them. Following a further PBS wash, the cells were then resuspended in 70% ethanol for 15 min. After aspiration of the ethanol, 2 mL of liquefied Histogel (Richard-Allan Scientific) were added to the cell pellet dropwise. The Histogel was gently vortexed and cooled on ice until solid. Histogel pellets were then processed in a tissue processor in the usual way.

Immunofluorescence

For immunofluorescence experiments, 5 × 10^4 cells were seeded in four-well chamber slides (BD Biosciences) for 24 h. Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min at room temperature, and permeabilized with PBS/0.1% Triton X-100 for 5 min at room temperature. After two washes with PBS, cells were blocked with PBS/1% BSA for 1 h followed by incubation with the primary antibody diluted in PBS/1% BSA for 1 h at room temperature. Cells were rinsed three times with PBS and then incubated with Texas red antimouse IgG (Vector Laboratories) for 1 h at room temperature, and the cells were washed three times. Finally, the chamber slides were stained with 4′,6-diamidino-2-phenylindole and mounted with Vectashield mounting medium (Vector Laboratories). Cells were imaged at ×20 with a Plan-Apochromat oil immersion lens on an Axioplan 2 Apoptome epifluorescence microscope (Zeiss).

Immunohistochemistry and scoring

Immunohistochemistry was done on 5 μm thick, formalin-fixed, paraffin-embedded tissue microarray and full sections using a primary antibody for FOXA1, p27kip1, and MIB-1. The primary antibody was diluted 1:100 or 1:200 and incubated for 60 min at room temperature. Antigen retrieval was done for three successive 5 min microwave cycles in EDTA (pH 8). The BioGenex I 6000 autostainer was used for all subsequent steps (BioGenex). Primary antibody was detected using a biotin-avidin-conjugated secondary mouse antibody (1:200; Vector Laboratories), horseradish peroxidase and 3,3′-diaminobenzidine. Counterstaining was with hematoxilin.

Positive controls included human prostate tissues and MCF-7 cell pellets were run with each assay.

Nuclear staining for FOXA1, p27kip1, and MIB-1 was assessed quantitatively by using the following scoring methods: (a) quantity: 1, negative; 2, 1% to 10% of examined nuclei positive (low expression); 3, 11% to 50% of nuclei positive; and 4, >50% of nuclei positive (high expression).
expression). Scores were averaged across replicate cores. (b) Intensity: 0, negative; 1, weak; 2, moderate; and 3, strong.

Positively stained lymphocytes represented the internal control for p27kip1 immunohistochemistry.

**Cytogenetic analysis**

**Slide preparation.** Sections (4 μm) of formalin-fixed, paraffin-embedded tumors were mounted on standard glass slides. Slides were baked at 60°C for at least 2 h and soaked for 15 min in xylene at 55°C and then in room temperature xylene for 15 min. Slides were dehydrated, air dried, boiled in 100 mmol/L Tris, 50 mmol/L EDTA (pH 7.0) for 1 h, and soaked in 2× SSC for 5 min. Slides were placed on a 37°C Thermobrite (StatSpin) and treated with Digest-All III solution (Invitrogen) for two 15 min digestions, fixed for 2 min in 10% phosphate-buffered formalin, and then dehydrated.

**Fluorescence in situ hybridization.** Each BAC DNA (1 μg) was labeled using a nick translation kit (Abbott Molecular/Vysis) following the manufacturer's directions. Labeled DNA was precipitated with 5 μL Cot-1 DNA (1 mg/mL stock) and resuspended in an appropriate volume of 50% Hybrisol (50% formamide, 2× SSC, 10% dextran sulfate). Slides and probes were denatured according to standard protocols. Hybridizations were done for at least 16 h at 37°C in a darkened humid chamber. Slides were washed in 2× SSC at 70°C for 10 min, rinsed 2× SSC at room temperature, and counterstained with 4',6-diamidino-2-phenylindole II (Abbott Molecular/Vysis). Slides were imaged using an Olympus BX51 fluorescence microscope, and individual images were captured using an Applied Imaging system running CytoVision Genus version 3.9.

Probes-BAC clones RP11-314P15 (chromosome 14q12) and RP11-606C5 (spanning FOXA1 at 14q21.1) were labeled with Spectrum-Green and SpectrumRed dUTP, respectively (Abbott Molecular/Vysis). Both BAC clones were obtained from the BAC-PAC Resource at Children's Hospital Oakland Research Institute.

**Scoring.** For each specimen, at least 50 tumor nuclei were scored for the number of signals corresponding to the FOXA1 (T, test probe) and control probe (C), respectively, and T:C ratios were determined. Nuclei with a T:C ratio of 1:1 but with >2 signals per locus were scored as polysomic. Nuclei with a T:C ratio of >1 but with <3 signals per locus were scored as having FOXA1 copy number gain. Nuclei with a T:C ratio >3 were scored as amplified for FOXA1.

**Statistical analysis**

Results were compared by Student’s t test (*, P < 0.05; **, P < 0.01; ***, P < 0.001). FOXA1 and p27kip1 protein expression levels were compared by Pearson correlation coefficient. Statistical analysis was carried out with Microsoft Excel software.

**Results**

**FOXA1 overexpression in ATC.** To investigate whether FOXA1 is involved in thyroid tumorigenesis, we first examined FOXA1 mRNA and protein expression levels in human thyroid cancer cell lines. Several of the commonly used human thyroid cell lines have been recently shown to be not only redundant (cross-contaminated) but also of nonthyroid origin likely arising from melanoma and primary human colon carcinoma (23). For this reason, we performed our experiments on those cells with confirmed authenticity (23).

We examined the expression of FOXA1 by Western blot (Fig. 1A) and immunofluorescence (Fig. 1B-D) in 8505c cells, an undifferentiated thyroid carcinoma cell line, WRO82-1 cells, a follicular carcinoma cell line, and BCPAP and TPC-1 cells, papillary carcinoma cell lines. Whereas WRO82-1, BCPAP, and TPC-1 were negative for FOXA1 expression (data not shown), FOXA1 expression was detected in 8505c and WRO82-1 cells.

![Fig. 2. FOXA1 expression in human primary thyroid undifferentiated carcinomas cells (ATC) and FISH analysis (all magnification, ×40). H&E stains of ATC cases showing squamoid morphology (A), classic giant cell and spindle cell morphology (D). B and E, intense nuclear FOXA1 expression in ATC cells by immunohistochemistry. Note the high mitotic activity (arrows). FOXA1 copy number gain by polysomy was observed in interphase tumor nuclei of ATC specimens IC and P1 (green) expressing high protein levels by immunohistochemistry (B and E). RP11-314P15 BAC clone (red) was cohybridized with a control RP11-606C5 BAC clone (green). C and F, representative sections from ATC with copy number gains of FOXA1. Intense FOXA1 nuclear staining is detected by immunohistochemical analysis (B and E).](www.aacrjournals.org)
shown), 8505c cells expressed high levels of FOXA1, which was exclusive nuclear.

We confirmed the specificity of the anti-FOXA1 antibody by infecting 8505c cells with two lentiviral vectors expressing shRNAs specific for FOXA1 (sh#1 and sh#2) or the control anti-GFP shRNA. FOXA1 mRNA levels were decreased ∼30% by sh#2 and by >90% by sh#1 (Fig. 1G). This degree of silencing of the FOXA1 mRNA was reflected by a similar level of silencing of the protein (Fig. 1H), confirming both the specificity of the anti-FOXA1 antibody and the effectiveness of the shRNAs.

We next asked whether FOXA1 expression could be detected in primary ATC. Differentiated thyroid carcinomas and insular thyroid tumors were negative for FOXA1 as were normal thyroid tissue and benign lesions. Human ATC, which are characterized by marked cellular polymorphism (giant, spindle, and squamoid cells), eccentric nuclei, and conspicuous nucleoli, showed nuclear overexpression of FOXA1 protein (nuclear staining score 3 or 4; Table 1). FOXA1 was expressed diffusely within the tumors with moderate to strong intensity (overexpression) in 14 of 20 (70%) ATC cases (Fig. 2), whereas 4 of 20 (20%) showed focal and low expression and 2 of 20 (10%) were negative. Strong nuclear expression was mainly observed in ATC with squamoid or squamous growth pattern characterized by markedly atypical nuclei and high proliferative activity as shown by the presence of numerous atypical mitoses (Fig. 2) and high MIB-1 proliferative index (>85%; Supplementary Fig. S1A). Moreover, we found moderate to strong nuclear expression of FOXA1 in foci of squamous metaplasia of 2 cases of papillary thyroid cancer.

Finally, 7 ATC arising in association with small foci of differentiated papillary thyroid carcinoma showed FOXA1 expression only in the undifferentiated areas (Supplementary Fig. S1B). Differentiated papillary thyroid carcinomas areas showed a very weak nuclear and cytoplasmic expression barely above the threshold of detection and not comparable with the expression levels seen in ATC. These weakly expressing cases were considered negative for FOXA1 expression (Supplementary Fig. S1C).

**Genomic gain of FOXA1 in human primary ATC specimens.** To determine whether FOXA1 expression in ATC was linked to FOXA1 copy number gain, we performed fluorescence in situ hybridization (FISH) analysis. FISH results were obtained for 14 of 20 ATC specimens because suboptimal tissue fixation precluded successful FISH analysis of six specimens. Hybridization of BAC RP11-606C5 to the TE4 cell line (human esophageal squamous carcinoma cell line with amplification of FOXA1) was used as positive control and confirmed a pattern of amplification consistent with a homogeneously staining region. A range of three to seven copies of FOXA1 was detected in 10 of 14 (71.4%) ATC specimens (Fig. 2) and occurred as a result of polysomy (hybridization pattern consistent with multiple copies of chromosome 14). Disomy for FOXA1 was observed for 2 of 14 (14%) ATC specimens, with a signal pattern consistent with monosomy 14 detected in the remaining 2 of 14 ATC specimens. In addition, 8 of 11 (72.7%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2), 2 of 11 (18.1%) ATC tumors with nuclear FOXA1 protein overexpression showed copy number gain (Fig. 2). Finally, 8505c cells showed copy number gain of FOXA1 due to polysomy (Supplementary Fig. S2C). In summary, we show that FOXA1 is overexpressed and subject to copy number gain in human ATC.

![Fig. 3 Effects of FOXA1 silencing on cell cycle of human thyroid carcinoma cells.](image)
Down-regulation of FOXA1 suppresses cell proliferation in human ATC cells correlated with transcriptional up-regulation of p27kip1. We investigated the functional role of FOXA1 in ATC cells by examining the effect of FOXA1 silencing on cell cycle progression and cellular proliferation. Indeed, it has been previously shown that poorly or undifferentiated human thyroid cancer cells exhibit considerable imbalance of their cell cycle and multiple abnormal cell signal transduction pathways.
that determine rapid cellular proliferation, reduced apoptosis, and genomic instability (24).

To answer the question whether FOXA1 may affect cell proliferation, we conducted a cell cycle analysis in 8505c cells after FOXA1 silencing by two independent shRNAs. Cell growth curves after 1 week of FOXA1 showed a strong decrease of 8505c cellular proliferation by sh#1 ($P < 0.01$) and a slight decrease by sh#2 ($P < 0.05$; Fig. 3A), which were consistent with the levels of FOXA1 silencing (Fig. 1G-H). Similarly, BrdUrd incorporation (% of cell uptake) decreased in shRNA-treated cells compared with the control (sh#1 $P < 0.01$ and sh#2 $P < 0.05$; Fig. 3B and C).

We further investigated the cell cycle distribution of 8505c cells by flow cytometry analysis in which FOXA1 had been silenced and observed a reduction of the fraction of cells in S phase (Fig. 3D and E), concomitant with an increase in the number of cells in both G1 ($P < 0.001$) and G2-M ($P < 0.05$) phases. The G1-S transition of the cell cycle is controlled by different cyclins. Hence, we monitored the effect of FOXA1 silencing on several of these molecular factors: p21waf1, cyclinD1, cyclinE1. None of these cyclins was differentially expressed between sh-FOXA1 and sh-control in 8505c cells at either mRNA or protein level (Supplementary Fig. S3A and B).

However, we found that p27kip1, a crucial cell cycle inhibitor, was significantly increased in 8505c cells infected with either FOXA1 sh#1 or sh#2 at both mRNA and protein levels (Fig. 3F and G).

Finally, FOXA1 silencing did not induce apoptosis in the infected cells as confirmed by flow cytometry analysis (absence of sub-G1 cell population; Fig. 3D and E) and absence of cleaved poly(ADP-ribose) polymerase immunoblot (Supplementary Fig. S3B).

**Inverse correlation between p27kip1 and FOXA1 expression in human ATC specimens.** p27kip1 expression was investigated by immunohistochemistry in 20 human ATC cases. Two cases were excluded due to technical issues. The remaining 18 cases were divided into four categories with respect to p27kip1 and FOXA1 expression and an inverse correlation between these protein levels was observed ($r = -0.547$; Fig. 4). Eleven (61%) of the cases had high nuclear FOXA1 expression (3 or 4+) and absent p27kip1 expression (1+; Fig. 4). Two (11%) of the cases had absent FOXA1 expression (1+) and moderate to high p27kip1 expression (2 or 3+; Fig. 4). Four (22%) of the cases had low FOXA1 expression (2+) and absent p27kip1 expression (1+; Fig. 4). One (6%) case had absent FOXA1 and p27kip1 expression (1+ for both; Fig. 4). In addition, 6 of 18 (33.3%) ATC showed a residual small focus of differentiated papillary thyroid carcinoma and weak p27kip1 cyttoplasmic expression was observed in all differentiated tumor foci associated with ATC (Supplementary Fig. S4).

**Discussion**

In this study, we found that human ATC express FOXA1 protein and show an intriguing link between FOXA1 expression, undifferentiated status, and cellular proliferation. Normal thyroid tissues, benign lesions, and differentiated thyroid carcinomas did not show detectable expression of FOXA1. Interestingly, we detected moderate nuclear protein levels of FOXA1 in squamous metaplasia foci of papillary well-differentiated thyroid carcinomas, a rare pathologic transformation that occurs in follicular thyroid tissues, still not well understood in terms of potential transition to undifferentiated thyroid cancer. This may suggest a role of FOXA1 in thyroid cell differentiation, because this transcription factor is already known to be expressed at 15.5 days in endoderm-derived mouse tissues including thyroid (25).

We found that FOXA1 nuclear overexpression was related to chromosome 14 gain in 72.7% of ATC. About 20% of the ATC that overexpressed FOXA1 showed low-level gain of FOXA1 (1-2 additional copies), consistent with unbalanced rearrangements involving this locus. Our results are supported by previous studies showing copy number gain of other chromosomes in ATC (26-28) due to ploidy changes or unbalanced rearrangements that significantly occur in aggressive papillary thyroid carcinomas (29) and poorly differentiated thyroid cancers (30) and are less frequent in well-differentiated papillary thyroid carcinomas (26). This evidence suggests that copy number gains are genetic events of the progression from well-differentiated to poorly differentiated thyroid cancers to ATC, as they are also associated with an aggressive clinicopathologic behavior.

FOXA1 has been shown to be amplified and overexpressed in 6% to 9% of human esophageal and 40% lung adenocarcinomas (31, 32). A copy number gain due to polysomy of the FOXA1 locus in ATC has not been reported. In our study, we found that 71.4% of ATC harbor this genomic alteration, supporting the idea that the genetic complexity of these tumors may play a role in producing the aggressive phenotype. We suggest that 14q gains are selected for because of FOXA1 overexpression, although we cannot rule out the possibility that this is simply the result of genomic instability. Conversely, we found a low percentage of disomic ATC in the FOXA1 locus, suggesting that other potential genetic alterations could occur. There was one ATC specimen (<10%) that showed FOXA1 overexpression and monosomy of chromosome 14.

We showed that shRNA-mediated silencing of FOXA1 significantly suppresses proliferation by blocking the G1-S transition of anaplastic thyroid cancer cells. These data suggest that FOXA1 might be one of the genes necessary and involved in the complex machinery of cellular proliferation of anaplastic thyroid cancer cells and could represent a potential new target for treatment of undifferentiated thyroid cancers. We
propose that inhibition of cell proliferation due to FOXA1 silencing in undifferentiated thyroid carcinoma cells resulted in the reexpression of p27kip1 mRNA and protein levels, which are usually very low or absent in this type of cancer. This is supported with the inverse correlation between loss of nuclear p27kip1 protein and high levels of FOXA1 protein in human ATC cells. Our results suggest that FOXA1 in ATC cells could be a novel and important gene affecting cell proliferation by a potential transcriptional inhibition of p27kip1. A FOXA1 binding site within the BRCA1-responsive element of the p27kip1 promoter has been identified (33). p27kip1 has been widely known to be a critical inhibitor of cell cycle progression in human cancer cells (34), including thyroid cancer cells (35).


profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. J Clin Endocrinol Metab 2008;93:4331–41.


Carmelo Nucera, Jerome Eeckhoute, Stephen Finn, et al.


**FOXA1 Is a Potential Oncogene in Anaplastic Thyroid Carcinoma**

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