Copy Number Aberrations of Genes Regulating Normal Thymus Development in Thymic Epithelial Tumors

Iacopo Petrini¹, Yisong Wang¹, Paolo A. Zucali³, Hye Seung Lee¹,⁴, Trung Pham¹, Donna Voeller¹, Paul S. Meltzer², and Giuseppe Giaccone¹

Abstract

**Purposes:** To determine whether the deregulation of genes relevant for normal thymus development can contribute to the biology of thymic epithelial tumors (TET).

**Experimental Design:** Using array comparative genomic hybridization, we evaluated the copy number aberrations of genes regulating thymus development. The expression of genes most commonly involved in copy number aberrations was evaluated by immunohistochemistry and correlated with patients’ outcome. Correlation between FOXC1 copy number loss and gene expression was determined in a confirmation cohort. Cell lines were used to test the role of FOXC1 in tumors.

**Results:** Among 31 thymus development-related genes, PBX1 copy number gain and FOXC1 copy number loss were presented in 43.0% and 39.5% of the tumors, respectively. Immunohistochemistry on a series of 132 TETs, including those evaluated by comparative genomic hybridization, revealed a correlation between protein expression and copy number status only for FOXC1 but not for PBX1. Patients with FOXC1-negative tumors had a shorter time to progression and a trend for a shorter disease-related survival. The correlation between FOXC1 copy number loss and mRNA expression was confirmed in a separate cohort of 27 TETs. Ectopic FOXC1 expression attenuated anchorage-independent cell growth and cell migration in vitro.

**Conclusion:** Our data support a tumor suppressor role of FOXC1 in TETs. Clin Cancer Res; 19(8); 1–12. ©2013 AACR.

Introduction

Thymic epithelial tumors (TET) are a group of rare neoplasms with heterogeneous histologic features and clinical behavior. Thymic carcinomas are aggressive tumors that microscopically remind the features of carcinomas of other organs (1). On the contrary, the histologic appearance of thymomas resembles the structure of normal thymus and these tumors are grouped into 5 subcategories (A, AB, B1, B2, B3), depending on their cancer cell shape, degree of atypia, and number of intratumoral thymocytes, according to the most recent World Health Organization (WHO) classification (1). Thymomas, but not thymic carcinomas, are frequently associated with paraneoplastic syndromes: myasthenia gravis being the most common (1). Surgery represents the mainstay of treatment for thymic malignancies, and prognosis is significantly influenced by pathologic stage and by completeness of tumor resection (2). Metastatic and nonresectable TETs are candidates for systemic therapy. Although, combination chemotherapy is able to induce substantial tumor shrinkage of variable duration, it is not curative in patients with metastatic disease (2). There is very little understanding of the biology of these neoplasms and molecular prognostic markers, and specific targets for therapy have so far not been identified.

Tumor cells share many stem cell-like properties with embryonic cells, and the ectopic reactivation of embryonic restricted genes is observed during the neoplastic progression (3). Several cell lineage-specific transcription factors, implicated in organogenesis, have been found to be ectopically reactivated by copy number aberrations in cancers, including NKX2-1 (TITF) in lung cancer (4–8), ESR1 in breast cancer (9), GATA6 in pancreatic cancer (10), and MITF in melanoma (11).

The role of thymic developmental genes in TETs has not been explored to date. Thymic epithelial cells originate from the endoderm of the third pharyngeal pouch (12). Finely spatiotemporally regulated waves of proliferation and differentiation of thymic epithelial cell precursors are necessary for the normal maturation of the thymus as well as...
Translational Relevance

Thymic epithelial tumors (TET) are a group of neoplasms with heterogeneous histologic features and clinical behavior. The identification of markers useful to predict patient prognosis and molecular targets for therapies is limited by a very little understanding of the biology of these neoplasms. We evaluated the copy number aberrations of genes involved in normal thymus development in TETs, following the intriguing idea that the ectopic deregulation of genes relevant for proliferation and differentiation of embryonic cells, can contribute to tumor growth. Frequent copy number losses of FOXC1 were observed in more aggressive tumors and correlated with a reduced protein expression; tumors negative for FOXC1 expression were associated with a shorter time to progression. In addition, FOXC1 showed tumor suppressor activity in in vitro models. Our data indicate that FOXC1 loss can identify a group of TETs with poor prognosis, possibly because of its tumor suppressor properties.

interactions with thymic septum cells of neural crest origin and lymphocyte precursors (thymocytes; ref. 13). To efficiently sustain this process, genes controlling the cell proliferation need to be expressed or repressed at precise moments of the development of thymic epithelial cells (13).

We conducted array comparative genomic hybridization (CGH) in TETs to determine the copy number status of genes involved in thymus development. Interestingly, FOXC1 and PBX1, two transcription factors that regulate TBX1 expression, were frequently included in regions of copy number loss and copy number gain, respectively. We suggest that FOXC1 presents tumor suppressor-like activity, and loss of FOXC1 expression correlates with poorer time to progression (TET) in patients with TET.

Materials and Methods

Our Institution Ethical Review Boards approved this research (ClinicalTrials.gov ID: NCT00965627). The study has been conducted in agreement with the Declaration of Helsinki.

Patients and samples

Cohort 1. Clinical data and formalin-fixed paraffin-embedded (FFPE) samples were collected from a series of 132 consecutive patients who underwent surgery for TET at the Istituto Clinico Humanitas (Rozzano-Milan, Italy) in the period from 1996 to 2008. A total of 109 patients underwent surgery for primary tumors and 23 for a tumor relapse. In case of relapsed tumors, the date of the first surgery was considered, extending the observation to a period ranging from 1976 to 2008. This series has been described in detail elsewhere (14). Patients were staged according to the Masaoka staging system (15). The completeness of resection was defined as R0 = complete resection, R1 = microscopic residual disease infiltrating resection margins, and R2 = macroscopic residual disease (16). An experienced thoracic tumor pathologist (H.S. Lee; Seoul National University Bundang Hospital, South Korea) reviewed the retrieved material for the amount of tumor content, adequate storage, and classified the TETs according to the 2004 WHO classification (1).

Confirmation cohort. Frozen TET samples from 27 patients were collected at National Cancer Institute (NCI; Bethesda, MD). Characteristics of patients are summarized in Table 1.

Nucleic acid extraction and array CGH

Because of the heterogeneity of the histologic features and the presence of non-neoplastic intrathoracic thymocytes, only 59 tumor samples containing at least 80% of tumor cells were selected for array CGH, as assessed by hematoxylin and eosin (H&E)-stained slides.

Frozen tumors were embedded in optimal cutting temperature and 8 µm slides were cut in a −20°C cryostat. Slides were stained with H&E, and sample regions with more than 80% of tumor cells were macrodissected for nucleic acid extraction.

From FFPE samples, DNA was extracted using DNeasy kit (Qiagen Inc.). Both RNA and DNA were extracted from cell lines and frozen tumors using AllPrep DNA/RNA Mini Kit (Qiagen). CGH was conducted using Agilent platform according to Genomic DNA ULS labeling kit protocol (Agilent Technologies Inc.) as previously extensively described elsewhere (17). Twenty samples from FFPE were hybridized on Human Genome CGH Array 105A (Agilent) and the remaining on SurePrint G3 Human CGH Array 180K (Agilent). Frequency of copy number aberrations was inferred using Nexus Copy Number 6 (Biodiscovery Inc.). Microarray data have been deposited in gene expression omnibus repository with the number: GSE23540.

Copy number and real-time PCR

Copy number PCR was adopted to confirm CGH results in a subset of tumors. Real-time PCR (RT-PCR) was used for the evaluation of TBX1, PBX1, and FOXC1 gene expression in TET frozen tumors and cell lines. TaqMan gene expression assay primers were purchased from Applied Biosystems. RPLP0 and RPPH1 genes were used as endogenous controls for mRNA expression and gene copy number validation, respectively. RT-PCRs were operated on ABI 7900HT Fast RT-PCR System (Applied Biosystems). Fold change of mRNA expression was calculated by 2−ΔCt method, and gene copy number was determined by CopyCaller software v1.0 (Applied Biosystems). For copy number assay, normal male diploid genomic DNA (Promega) was used as a reference after 10 minutes heat fragmentation at 99°C. For copy number PCR, because FOXC1 contains only one exon, 2 couples of primers were designed to avoid possible issues related to residual RNA contamination: one covering the exonic sequence and the other one covering an intergenic region on the chromosome 6p.
**FOXC1 sequencing**

Primers used for sequencing of the FOXC1 gene are available upon request. Primers were tagged with M13 forward or M13 reverse sequences. DNA from FFPE samples were amplified by PCR using AmpliTaq Gold PCR Master Mix (Applied Biosystems) and Veriti 96-Well Thermal Cycler (Applied Biosystems). PCR products underwent ExoSAP-IT (USB) purification. The purified products were directly sequenced using a BigDye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems) and 3730xl DNA Analyzer (Applied Biosystems). Data were analyzed using Mutation Surveyor v 3.23 (SoftGenetics LLC).

**Immunohistochemistry**

Immunohistochemistry was conducted using a tissue microarray that included samples from 132 patients with TET, as previously described (14, 18). Anti-FOXC1 (1:200, ab5079; Abcam) and anti-PBX1 (1:150, HPA003881; Sigma) antibodies were used. According to the human protein atlas website, normal human kidney and pancreas were used as controls for FOXC1 and PBX1, respectively. Positive controls were stained with anti-FOXC1 and anti-PBX1 antibodies and negative controls using respective isogenic serum instead of the primary antibodies. Both percentage and intensity of the stained cells were taken into consideration for immunohistochemistry scoring. Percentage of positive cells was ranked as 0 (0%–5%), 1 (6%–25%), 2 (26%–50%), 3 (51%–75%), or 4 (76%–100%). For FOXC1, signal intensity 0 to 1 was considered negative and 2 to 3 positive; based on the rank of positive cells, samples were graded G0 (rank 0), G1 (rank 1), G2 (rank 2), or G3 (rank 3 and 4). For PBX1, signal intensity was multiplied by rank of positive cells and scores from 0 to 8 were considered negative (G0), scores of 9 were G1, 10 to 11 were G2, and 12 to 13 were G3.

**Cell lines and experiments**

T1889 (thymic carcinoma) and T1682 (B1 thymoma) cell lines were kindly provided by Dr. Marco Breinig (Institute of Pathology, Heidelberg University Hospital, Heidelberg, Germany; ref. 19). TY82 thymic carcinoma cell line was purchased from Japan Health Science Foundation (Tokyo), whereas, NIH-H82, NIH-H69, NIH-H23, NIH-H460, NIH-H1355, HEK-293, NIH-3T3, and U2OS were obtained from American Type Culture Collection. NIH-3T3 cells were cultured in Dulbecco’s modified Eagle medium and all other cell lines were cultured in RPMI-1640; media were supplemented with 200 mmol/L glutamine (Invitrogen), 50 U/mL penicillin, 50 U/mL streptomycin (Invitrogen), and 10% heat-inactivated calf serum (Invitrogen) and grown in a 37°C incubator with humidified 5% CO2 atmosphere. For T1889 and T1682, medium was supplemented with 25 nmol/L Hepes.

**Western blot analysis**

Protein extraction and Western blot analysis were conducted as previously described (20, 21) using anti-N-terminal-FOXC1 (1:200, overnight 4°C incubation; Santa Cruz biotecnology Inc.), anti-PBX1 (1:1,000, overnight 4°C incubation; Cell Signaling Technology), anti-TBX1 (1:1,000, overnight 4°C incubation; Epitomics), and anti-α-Tubulin (1:2,000, 1-hour room temperature incubation, Cell Signaling) antibodies, respectively.

**Statistical analysis**

Survival curves were generated using the Kaplan–Meier method. Disease-related survival (DRS) was determined from the date of surgery on primary tumor to the date of death due to tumor progression. TTP was calculated from date of surgery to relapse or progression. Differences between survival curves were, first, determined by a log-rank test. Subsequently, those factors, which seemed to be at least modestly associated with outcome (P < 0.10) in univariate analyses were evaluated for their joint impact on TTP or DRS using a Cox proportional hazard model. All P values are 2-tailed and have not been adjusted for multiple comparisons. Clinical and biologic characteristics were compared using 2-tail Fisher exact test or χ2 test or Student t test or one-way ANOVA with Tukey test for post hoc comparisons, when appropriate. Pearson R coefficient described the correlation between FOXC1 and TBX1 expression. All tests were conducted using the SPSS version 17 (SPSS, Inc.), SAS version 8.2 (SAS Institute Inc.), and Prism 5 (GraphPad Software).

**Results**

**Copy number aberrations of genes involved in thymus development**

A group of 31 genes implicated in the thymus organogenesis was identified from an extensive literature review (12, 13, 22, 23). The copy number aberrations of these 31 thymic developmental genes were determined using array CGH in a series of 59 FFPE TET samples. Frequencies of copy number aberration are summarized in Fig. 1. The most frequent copy number alterations were copy number gains of PBX1 and GLI4, as well as copy number losses of FOXC1 and IGF2R (P < 0.05). Interestingly, both PBX1 and FOXC1 have been described to be able to regulate the expression of TBX1, a transcription factor indispensable for the thymus development.

FOXCI copy number loss was found in a narrow region of copy number loss affecting about 2 Mb (1,996,266 nucleotides) of the short arm of chromosome 6 (Fig. 2A). On the basis of our CGH data, chr6p25 copy number loss, where FOXC1 resides, was observed in all histotypes, including type A thymomas that usually present infrequent copy number aberrations (Fig. 2B). The copy number loss of chromosome 6p has also been described in another case of A thymoma by Zetel and colleagues (24). However, chr6p25 copy number loss was less commonly observed in A thymomas (8%) than in other histotypes (40%; Fisher exact test; P = 0.044; Fig. 2B). FOXC1 copy number loss was observed in 27% of stage I–II and in 35% of stage III–IV patients, and the difference was not significant. Similarly, 30% of completely resected cases and 35% of cases with residual disease (R1+R2) carried FOXC1 copy number loss.
In contrast, PBX1 locus was included in a large region of copy number gain involving the entire chromosome 1q (Fig. 2C). PBX1 copy number gain was a more frequent event in aggressive histotypes (B2+B2/B3, B3, and thymic carcinoma) than in A and AB (Fisher exact test, $P < 0.001$; Fig. 2D), and in relapsed tumors than in primary tumors ($\chi^2$ test, $P = 0.027$).

Copy number aberrations of thymic developmental genes were confirmed using array CGH in an independent cohort of 27 frozen TET samples collected at National Cancer Institute (Bethesda, MD). Also in this series, PBX1 (59.3%) and GLI4 (37%) were the most frequent copy number gains and FOXC1 (51.9%) and IGF2R (33.3%) were the most frequent copy number losses (Supplementary Fig. S1 and Table 1). FOXC1 copy number loss was present in 20% of type A thymoma and in 59.1% of the other histotypes (Fisher exact test, $P = 0.165$). Also in the confirmation cohort, PBX1 copy number gain was more frequent in more aggressive histotypes (75%) than in A and AB tumors (14%; Fisher exact test, $P = 0.009$).

Across the 2 series (86 tumors), FOXC1 copy number loss was observed in 34 tumors (39.5%) and PBX1 copy number gain in 37 (43.0%; summary of combined data are reported in Supplementary Fig. S2, S3A, and S3B).

**FOXCI copy number loss and PBX1 copy number gain technical validation**

Technical validation of CGH results was conducted using copy number PCR. Chromosome 6p23.5 (FOXC1) deletion was evaluated in 6 tumors with and 5 tumors without copy number loss, according to CGH results. Copy number loss was confirmed in 5 of 6 tumors that showed FOXC1 copy number loss by CGH, whereas, in the control group,
without copy number loss of FOXC1, CGH and copy number PCR data were 100% concordant (Fisher exact test, \( P = 0.015 \)). Copy number gains of \( PBX1 \) locus were confirmed in all tested samples: 7 with and 7 without CGH gain (Fisher exact test, \( P < 0.001 \)).

**FOXC1 and PBX1 expression in TETs**

We explored the correlation between copy number status and protein expression using immunohistochemistry conducted on a tissue microarray containing 132 tumors that included the 59 samples evaluated by array CGH.

Of 119 TET cases evaluable for FOXC1 immunostaining, 94 (79%) were negative (Table 2). In positive cells, staining was predominately nuclear according to the nature of FOXC1 that is a transcription factor (Fig. 2E and F). Mainly tumor epithelial cells were stained but not thymocytes. Only epithelial cells were considered in scoring the intensity of FOXC1 staining. A total of 81.8% of A thymoma were strongly positive for FOXC1 staining: all the positive cases were classified as G3. Overall 90% of the tumors strongly expressing FOXC1, with a score of G3, were A and AB. In B histotypes, FOXC1 expression was uncommon, and when present, it was commonly with a reduced intensity. None of the 13 evaluable thymic carcinomas showed positive FOXC1 staining. FOXC1-negative staining was associated with worse patient outcome and tumor characteristics: relapsed tumors, incomplete resections, and more aggressive histotypes (Table 2). There was a significant correlation between FOXC1 copy number loss and immunohistochemistry results (\( P = 0.008 \)): 90% of tumors with FOXC1 copy number loss did not express FOXC1, whereas only 55% of tumors with 2 copies of FOXC1 were negative for its expression. FOXC1-negative cases were associated with worse TTP than FOXC1-positive cases (10-year TTP 62% vs. 93%, respectively; log-rank, \( P = 0.026 \); Fig. 2G). A similar trend was observed for DRS (log-rank, \( P = 0.139 \); Fig. 2H).

However, multivariate models could not be constructed for DRS because there were no death events observed in FOXC1-positive patients. FOXC1 was not an independent prognostic factor for TTP (\( P = 0.15 \); HR, 0.23; 95% confidence interval (CI) on HR, 0.03–1.73) after adjusting for WHO histotype (\( P = 0.0047 \); HR, 4.02; 95% CI on HR, 1.53–10.55) in a Cox multivariate analysis.

No correlation was observed between copy number gain of the \( PBX1 \) locus and \( PBX1 \) protein expression by immunohistochemistry (\( P = 0.575 \)). Moreover, there was no statistically significant difference in DRS or TTP between \( PBX1 \)-positive and negative cases (log-rank, \( P = 0.294 \) and \( P = 0.229 \), respectively). \( PBX1 \) expression was higher in more advanced stages (III and IV: 50%; 14/28) than in stages I and II (12.7%; 9/72; Fisher exact test, \( P < 0.001 \)). In advanced stages, \( PBX1 \)-positive patients had a better outcome (10-year DRS 100%; 5-year TTP 100%) than \( PBX1 \)-negative cases (10-year DRS 67%; log-rank, \( P = 0.058 \); 5-year TTP 42%; log-rank, \( P < 0.001 \)). More aggressive histotypes (B1/B2, B2/B3, B3, and thymic carcinoma) exhibited higher frequency of \( PBX1 \) expression (42.9%; 24/56) than less aggressive ones (A, AB, B1; 13.6%; 8/59; Fisher exact test, \( P = 0.001 \)). In the subgroup of more aggressive histotypes, \( PBX1 \)-positive cases had a better outcome (10-year DRS 94%; 10-year TTP 72%) than negative cases (10-year DRS 74%; log-rank 0.069; 10-year TTP 36% log-rank, \( P = 0.01 \)).

### Sequencing of FOXC1 gene

Because FOXC1 gene mutations have been shown in endometrial and ovarian cancers (25), we sequenced the entire FOXC1 coding region in the 59 FFPE TET samples analyzed by CGH and in the 3 TET cell lines. Three B3 samples showed an extraglycine insertion in a polyglycine region between amino acid 447 and 456. Normal DNA from 1 of the 3 patients was found to harbor this glycine insertion. One AB thymoma carried a glycine insertion in another polyglycine region between amino acid 375 and 380. No insertion at the same position was found in normal DNA from the same patient.

**FOXC1 and TBX1 mRNA expression do not correlate in TETs**

FOXC1 has been described to regulate TBX1 expression during embryogenesis, therefore, we studied FOXC1 and TBX1 expression using RT-PCR in the confirmation cohort because good quality mRNA was available.

We observed a reduced expression of FOXC1 in tumors carrying FOXC1 copy number loss; however, the difference was not statistically significant (\( P = 0.263 \); Fig. 3A). This

### Table 1. FOXC1 copy number status and characteristics of patients of the confirmation cohort of 27 frozen TETs

<table>
<thead>
<tr>
<th>Number of cases</th>
<th>Frequency of FOXC1 copy number loss</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age</td>
<td>53</td>
</tr>
<tr>
<td>Range</td>
<td>36–76</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
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<tr>
<td>WHO histotype</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>5</td>
</tr>
<tr>
<td>AB</td>
<td>2</td>
</tr>
<tr>
<td>B2</td>
<td>5</td>
</tr>
<tr>
<td>B3</td>
<td>6</td>
</tr>
<tr>
<td>TC</td>
<td>9</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
</tr>
<tr>
<td>IIA</td>
<td>5</td>
</tr>
<tr>
<td>IIB</td>
<td>6</td>
</tr>
<tr>
<td>III</td>
<td>2</td>
</tr>
<tr>
<td>IVA</td>
<td>4</td>
</tr>
<tr>
<td>IVB</td>
<td>6</td>
</tr>
<tr>
<td>Na</td>
<td>4</td>
</tr>
</tbody>
</table>

Abbreviations: Na, stage at the diagnosis was not available for 4 patients; TC, thymic carcinoma.
trend \( (P = 0.085; \text{Fig. 3B}) \) was more evident considering exclusively thymomas, and excluding thymic carcinomas. FOXC1 copy number loss had no impact on the expression of TBX1 \( (P = 0.504; \text{Fig. 3C}) \), and also there was no correlation between FOXC1 and TBX1 expression (Pearson \( R = 0.062; 95\% \text{CI}, 0.325; 0.432; P = 0.757 \)). Interestingly, FOXC1 expression was more significantly pronounced in type A and AB tumors, and progressively decreased from B2–B3 to thymic carcinomas with the latter being almost completely negative for FOXC1 expression \( (\text{one-way ANOVA}, \ P = 0.001; \text{B2-B3 vs. thymic carcinoma, and thymic carcinoma vs. A-AB; Tukey test, } P < 0.05) \). There was no difference of TBX1 expression in different WHO histotypes of TETs \( (\text{one-way ANOVA}, \ P = 0.936) \).

FOXC1, a candidate tumor suppressor gene in TETs

According to the cancer cell line encyclopedia microarray expression data \( (26) \), positive (H23, H460, and H1355) and negative control lines (H69 and H82) were selected on the basis of FOXC1 expression. All TET cell lines, T1889 and TY82 thymic carcinoma and T1682 B1 thymoma, expressed FOXC1 \( (\text{Fig. 4A}) \). Array CGH
revealed that none of the TET cell lines had copy number loss of FOXC1 locus. These observations were confirmed by protein expression using Western blot analysis (Fig. 4B). Because none of the 3 TET lines was null for FOXC1 expression, the transfection of FOXC1 in these cells was not a suitable model to test its tumor suppressor properties. Therefore, we evaluated the effect of ectopic FOXC1 expression in U2OS (Fig. 4C–E) and NIH-3T3 cells (Fig. 4F). These cells were chosen over TET cells because they were negative for FOXC1 expression (Fig. 4C and F). If FOXC1 is a genuine tumor suppressor gene, reconstitution of FOXC1 into these cells should impede cell growth.

Table 2. Immunohistochemistry results and characteristics of patients

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Frequency of FOXC1 IHC+ %</th>
<th>P</th>
<th>Frequency of PBX1 IHC+ %</th>
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<tbody>
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<td>132</td>
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</tr>
<tr>
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<td>CGH results</td>
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<td>FOXC1 copy number loss</td>
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<td>10.0%</td>
<td>P = 0.008&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<tr>
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<td>P = 0.575</td>
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<td>PBX1 2 copies</td>
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<td>24.7%</td>
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<td>I</td>
<td>35</td>
<td>36.4%</td>
<td>P = 0.084</td>
<td>12.1%</td>
<td>P &lt; 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>I-II/III-IV</td>
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<td>III</td>
<td>19</td>
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<td>A</td>
<td>15</td>
<td>81.8%</td>
<td>P = 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
<td>36.4%</td>
<td>P = 0.001&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>AB</td>
<td>28</td>
<td>38.5%</td>
<td>A-AB-B1/</td>
<td>15.4%</td>
<td>A-AB-B1/</td>
</tr>
<tr>
<td>B1</td>
<td>24</td>
<td>0.0%</td>
<td>Remaining</td>
<td>0.0%</td>
<td>Remaining</td>
</tr>
<tr>
<td>B1/B2</td>
<td>6</td>
<td>16.7%</td>
<td></td>
<td></td>
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<tr>
<td>B2</td>
<td>8</td>
<td>0.0%</td>
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<tr>
<td>B2/B3</td>
<td>11</td>
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<td>B3</td>
<td>24</td>
<td>13.0%</td>
<td></td>
<td></td>
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<tr>
<td>TC</td>
<td>14</td>
<td>0.0%</td>
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<td></td>
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<td>Paraneoplastic syndromes</td>
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<tr>
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<td>91</td>
<td>25.6%</td>
<td>P = 0.205</td>
<td>21.0%</td>
<td>P = 0.017&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Yes</td>
<td>34</td>
<td>12.9%</td>
<td></td>
<td></td>
<td>45.2%</td>
</tr>
</tbody>
</table>

**Note:** Total number of cases is reported to describe characteristics of patients but frequency of FOXC1 and PBX1 staining is calculated on the number of evaluable cases: 119 and 117, respectively. IHC+, positive staining determined by immunohistochemistry; FOXC1 2 copies and PBX1 2 copies represent tumors without FOXC1 copy number loss and PBX1 copy number gain, respectively; Na, patients without available information at diagnosis; R0, complete resection; R1, microscopically incomplete resection; R2, macroscopically incomplete resection; TC, thymic carcinoma; Other, 1 micronodular and 1 cystic thymoma; Remaining, B1/B2, B2, B2/B3, B3 and TC.

<sup>a</sup>Significant differences calculated using Fisher exact test or χ² test when appropriate.
Soft agar assay showed that constitutive expression of FOXC1 in U2OS cells significantly reduced the number of colony outgrowth in comparison with the vector-transfected clones and U2OS parental cells (Fig. 4D and E). FOXC1 also attenuated the migration capacity of U2OS cells in Transwell migration assay (Fig. 4D). Intriguingly, stable clones derived from FOXC1-transfected NIH3T3 cells were all negative for ectopic FOXC1 expression (Fig. 4F) though FOXC1 was expressed in transiently transfected NIH3T3 cells (Fig. 4F), suggesting that FOXC1 reconstitution may be detrimental to the immortalized NIH3T3 cells, and only the clones capable of evading FOXC1 expression could grow. Collectively, we postulate that FOXC1 may be a candidate tumor growth suppressor gene in TETs.

Discussion

Recently, new technologies such as array CGH have enabled the evaluation of tumor-specific events on a genome-wide scale. Lineage-specific transcription factors, implicated in organogenesis, have been found deregulated by copy number aberrations and they have been shown to be able to drive the cancer phenotype. Examples include, NKX2-1 (TTF1) in lung cancer (4–8), ESR1 in breast cancer (9), GATA6 in pancreatic cancer (10), and MITF in melanoma (11). Using high-resolution array-based CGH, we have previously described the copy number aberrations of a series of 59 TETs on a genome-wide scale (17). Here, we have focused on the copy number aberrations of 31 genes implicated in thymus organogenesis (12, 13, 22, 23).
Subjects carrying the 22q11.2 deletion syndrome present variable pathologic phenotypes at birth including cardiac defects, abnormal faces, thymic hypoplasia, cleft palate, and hypocalcaemia. The concomitant presence of some of these congenital abnormalities has been named as specific syndromes: for example DiGeorge, velocardiofacial, and conotruncal anomaly face syndromes (27). Interestingly, several patients are immunocompromised because they were born with thymic aplasia or hypoplasia. TBX1, a member of the T-box containing family of transcription factors, is mapped on 22q11.2, and it is the more likely candidate responsible for the pharyngeal arch-derived defects observed in 22q11.2 deletion syndrome (27). TBX1 knockout mice showed the importance of this gene in the congenital branchial arch pathologic development and consequently in the maturation of the epithelial compartment of the thymus that originates from the endoderm of the third pharyngeal pouch (28).

TBX1 exerts its activity integrated in a complex cascade of transcription factors that regulates thymus development. It has been shown that FOXC1 and PBX1 transcription factors regulate TBX1 expression in vitro and in animal models (29, 30), acting downstream of Sonic Hedgehog (SHH) pathway during thymus development (13, 30, 31). TBX1 transcription is tightly spatiotemporally regulated by the SHH pathway and by HOX-EYA-PAX axis through FOXC1 and PBX1, respectively (30, 31). Upon the activation of SHH pathway, TBX1 is transcribed to regulate the expression of several genes, including FGF10 and FGF8 that support growth of surrounding cells and may also play a role in the migration of neural crest cells (32).

In vitro, TBX1 expression restores contact inhibition and reduces anchorage-independent cell growth in soft agar (33).

Among the 31 genes regulating thymus development, FOXC1 and PBX1 were those with the highest frequency of copy number loss and gain in TETs, respectively. Copy number gain of PBX1 is frequently observed in Tumorscape, and has been described in 35% of all cancer analyzed, but it
is often associated to a large region of copy number aberration (34). FOXC1 copy number loss is associated with a focal peak of deletion and less frequently observed in common cancers (12%, Tumorscape) than in TETs (39.5%). However, some types of tumors presented a remarkably high frequency of FOXC1 copy number loss, the highest frequency being 89% in colorectal cancer (34). In contrast, copy number gain of FOXC1 was observed in a large region of copy number gain of the whole chromosome 1q, being 1q copy number gain a frequent event in TETs (17, 24). Therefore, it is difficult to link the deregulation of a single gene to this large region of copy number gain (Fig. 2C). On the contrary, in this study, we observed a frequent FOXC1 copy number loss in a focal deletion including a limited number of genes. Copy number aberrations often affect the expression of the genes mapped to that region. Therefore, we correlated the protein expression of FOXC1 and PBX1, evaluated by immunohistochemistry, with their copy number status. FOXC1 expression was reduced in tumors with copy number loss, but PBX1 expression was not increased in tumors with copy number gain. Consistently, in the confirmation cohort, tumors with FOXC1 copy number loss expressed less FOXC1 mRNA. Because there was no correlation between PBX1 expression and FOXC1 copy number gain, we suggest that FOXC1 copy number loss but not PBX1 copy number gain may play a role in TET development.

It is important to note that FOXC1 staining was negative in 90% of tumors carrying FOXC1 copy number loss and 55% (20/36) of tumors with 2 FOXC1 alleles. Similarly, a very low expression of FOXC1 mRNA was observed in many tumors without FOXC1 copy number loss (Fig. 3A). Therefore, the copy number status of FOXC1 is probably not the exclusive factor influencing FOXC1 expression. Probably, there are other mechanisms in thymic carcinomas and to some extent in B2 and B3 thymomas that inhibit FOXC1 expression. Indeed, FOXC1 was not an independent prognostic factor in the multivariate analysis after correction for WHO histotype. We excluded the possibility of FOXC1 mutation as cause of low expression by conventional sequencing in 59 cases. The extraglycine insertions in polyglycine regions (of 6- and 10-glycine) were observed in 3 tumors and have been previously described as polymorphisms, thus unlikely affecting the protein function. Methylation of FOXC1 promoter is a candidate mechanism for repression of FOXC1 expression in tumors without FOXC1 copy number loss, as FOXC1 promoter methylation has been described in other cancer types and correlated with FOXC1 silencing (35). A poor outcome of FOXC1-negative tumors has been observed in invasive ductal breast carcinoma and locally advanced breast cancer (36, 37), though contradictory results have also been reported (34).

Because of the FOXC1 role in regulation of TBX1, we studied the association between FOXC1 and TBX1 mRNA expression. There was no significant correlation in tumors of the patient (Fig. 3D) or TET cell lines. Moreover, we failed to find any correlation between PBX1 copy number gain and TBX1 expression (data not shown). Therefore, TBX1 seems to be regulated in a more complex manner than solely by FOXC1. To evaluate the functional significance of FOXC1 deletion in TETs, we reconstituted FOXC1-negative U2OS cells, and showed that ectopic FOXC1 attenuated anchorage-independent growth and motility of U2OS cells in vitro, suggesting that FOXC1 may act as a tumor growth suppressor gene. In line with these findings, we were not able to establish a stable clone of NIH3T3 cells transfected with FOXC1. Because of the small number of TET cell lines generated to date, only 3 were available to us. Unfortunately, all TET cell lines expressed FOXC1; thus, tumor suppressor activity of FOXC1 in TET cell lines cannot be tested.

Our FOXC1 results in TETs and in U2OS cells are discrepant to what has been described in basal-like breast cancer, where FOXC1 acts as a candidate oncogene. FOXC1 overexpression has been described in basal-like breast cancers where overexpression was a poor prognostic factor (38). Ectopic FOXC1 overexpression in breast cancer cell lines increases cell proliferation, invasion, and migration (38). It has been suggested that FOXC1 may induce epithelial–mesenchymal transition (39), and consequently may increase the metastatic potential (39). Because FOXC1 is a transcription factor, epigenetic context of FOXC1 target genes may determine FOXC1 accessibility to their regulatory binding sites in the promoter sequences. Because thymic epithelial cells and precursors of basal-like breast cancer undergo unique differentiating programs, chromatin conformation is expected to be different for thymic epithelial cells of endodermic origin and mammary glands cells of ectodermic origin. Therefore, different promoters will be accessible to FOXC1 in these 2 different contexts with possibly different effects on cancer cell growth. Frequently, the altered expression of transcription factors, usually restricted to normal cell precursors, requires a specific intracellular context to restore their function and to induce proliferation. It is possible that such intracellular context is present exclusively in the committed line of descendant cells, indicating a state of "lineage dependency" (11).

In conclusion, we identified frequent copy number loss of FOXC1 in TETs, especially in more aggressive histotypes. A reduction of FOXC1 expression was observed in tumors with FOXC1 copy number loss and was associated with poor prognosis. In vitro data support the candidate tumor suppressor activity of FOXC1 on cell growth.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: I. Petrini, Y. Wang, P.A. Zucali, P.S Meltzer, G. Giaccone
Development of methodology: I. Petrini, H.S Lee, P.S Meltzer, G. Giaccone
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Petrini, P.A. Zucali, H.S. Lee, T.C. Pham, P.S Meltzer, G. Giaccone
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Petrini, P.A. Zucali, P.S Meltzer, G. Giaccone.

Writing, review, and/or revision of the manuscript: I. Petrini, Y. Wang, P.A. Zucali, P.S Meltzer, G. Giaccone.

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.A. Zucali, T.C. Pham, D. Voeller, P.S Meltzer.

Study supervision: Y. Wang, P.S Meltzer, G. Giaccone.

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References


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Iacopo Petrini, Yisong Wang, Paolo A. Zucali, et al.

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