Loss of the CBX7 Gene Expression Correlates with a Highly Malignant Phenotype in Thyroid Cancer

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Abstract

Using gene expression profiling, we found that the CBX7 gene was drastically down-regulated in six thyroid carcinoma cell lines versus control cells. The aims of this study were to determine whether CBX7 is related to the thyroid cancer phenotype and to try to identify new tools for the diagnosis and prognosis of thyroid cancer. We thus evaluated CBX7 expression in various snap-frozen and paraffin-embedded thyroid carcinoma tissues of different degrees of malignancy by quantitative reverse transcription-PCR and immunohistochemistry, respectively. CBX7 expression progressively decreased with malignancy grade and neoplasia stage. Indeed, it decreased in an increasing percentage of cases going from benign adenomas to papillary (PTC), follicular, and anaplastic (ATC) thyroid carcinomas. This finding coincides with results obtained in rat and mouse models of thyroid carcinogenesis. CBX7 loss of heterozygosity occurred in 36.8% of PTC and in 68.7% of ATC. Restoration of CBX7 expression in thyroid cancer cells reduced growth rate, with a retention in the G1 phase of the cell cycle, suggesting that CBX7 can contribute to the proliferation of the transformed thyroid cells. In conclusion, loss of CBX7 expression correlates with a highly malignant phenotype in thyroid cancer patients. [Cancer Res 2008; 68(16):6770–8]

Introduction

Thyroid tumors originating from follicular cells are a good model with which to investigate the events involved in carcinogenesis because they differ in malignant potential from differentiated to undifferentiated phenotypes (1, 2). Papillary thyroid carcinoma (PTC), which is differentiated and has a good prognosis, is the most frequent malignancy of the thyroid gland (2). The tall-cell variant (TCV) of PTC has a worse prognosis than conventional PTC (3). Poorly differentiated carcinomas (PDC) have a less differentiated phenotype and a poor 5-year survival rate (2). Finally, anaplastic carcinomas (ATC) are completely undifferentiated, very aggressive, and always fatal (2). In PTC, activation of the RET/PTC oncogene, caused by rearrangements of the RET proto-oncogene, occurs in about 20% of cases (2), whereas the B-RAF gene is mutated in about 40% of cases (4). These tumors have also been associated with TRK gene rearrangements (5) and MET gene overexpression (6). RAS gene mutations (7) and PAX8-PPAR-γ rearrangements (8) are frequent in follicular thyroid carcinomas (FTC), whereas impaired function of the p53 tumor suppressor gene is a feature of ATC (9). Although critical molecular mechanisms of thyroid carcinogenesis have been clarified, other molecular steps of thyroid neoplastic progression need to be investigated.

Therefore, within the context of a microarray study, we found that the CBX7 gene was down-regulated in thyroid carcinoma–derived cell lines and in surgically removed tumors. CBX7, which is located on chromosome 22q13.1, encodes a novel Polycarbonyl protein (Pc) of 28.4 kDa and 251 amino acids that contains a “chromodomain” between amino acids 10 and 46. The chromodomain was originally defined as a 37-amino-acid region of homology shared by heterochromatin protein 1 (HP1) and Pc proteins from Drosophila melanogaster (10, 11). The CBX7 protein seems to be involved in the control of normal cell growth (11, 12). Moreover, mouse CBX7 associates with facultative heterochromatin and with the inactive X chromosome, which indicates that CBX7 is involved in the repression of gene transcription (13).

Thyroid cancer is the most prevalent endocrine neoplasia. About 20,000 new cases are diagnosed in the United States each year, and >1,500 patients die of thyroid cancer annually. Given the poor prognosis associated with the less differentiated histologic types, namely TCV-PTC and Hurthle variants, and the undifferentiated ATC, there is a need for molecular markers that can help to predict prognosis, so that patients with a dismal prognosis can be offered a different, perhaps, innovative therapy.

In an attempt to determine whether CBX7 is related to thyroid cancer phenotype, and to find new tools for the diagnosis and prognosis of thyroid cancer, we have evaluated its expression in a large number of thyroid carcinoma tissues and in rat and mouse models of thyroid carcinogenesis. This analysis revealed a decreased CBX7 expression in an increasing percentage of cases going through from benign adenomas (FTA) to PTC, FTC, and ATC. Taken together, these results indicate a correlation of the loss of CBX7 expression with a highly malignant phenotype in thyroid cancer patients. Because restoration of CBX7 expression in thyroid cancer cells reduced growth rate, a role of the loss of CBX7 gene expression in thyroid carcinogenesis may be envisaged.
Materials and Methods

Cell culture and transfections. We used the following human thyroid carcinoma cell lines in this study: TPC-1, WRO, NPA, ARO, FRO, NIM 1, B-CPAP, FB-1, FB-2, Kat-4, and Kat-18, which are described elsewhere (14). They were grown in DMEM (Gibco Laboratories) containing 10% FCS (Gibco Laboratories), glutamine (Gibco Laboratories), and ampicillin/streptomycin (Gibco Laboratories) in a 5% CO₂ atmosphere. Normal human thyroid primary culture cells have been established and grown as already described (15). PC Cl 3 and FRTL-5 (17) cells were cultured in modified F12 medium supplemented with 5% calf serum (Gibco Laboratories) and six growth factors (thyrotropic hormone, hydrocortisone, insulin, transferrin, somatostatin, and glycy histidy llysine; Sigma). PC CL 3 and FRTL-5 infected with several oncogenes PC KiMSV, PC HaMSV, PC v-raf, PC MPSV, 16), PC PyVMV (18), PC E1A, PC E1A-v-raf (19), PC RET/PTC, PC HaMSV-RET/PTC1 (20), PC MPSV-HMG42 (21), FRTL-5 KiMSV (22), and FRTL-5 KiMSV-HMG1 (23) were cultured in the same medium as PC CL 3 and FRTL-5 cells but without the six growth factors. Thyroid cells were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer’s instructions. The transfected cells were selected in a medium containing genitin (G418; Life Technologies). For each transfection, several G418-resistant clones and the mass cell population were isolated and expanded for further analysis.

Human thyroid tissue samples. Neoplastic human thyroid tissues and normal adjacent tissue or the contralateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumors were collected at the Service d’Anatomo pathological, Centre Hospitalier Lyon Sud, Pierre Bénite, France. The tumor samples were stored frozen until required for RNA or protein extraction.

This study has been approved by the institutional review board of the Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli “Federico II”.

RNA isolation. Total RNA was extracted from tissues and cell cultures using the RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

Reverse transcription and PCR analysis. One microgram of total RNA from each sample was reverse-transcribed with QuantiTect Reverse Transcription Kit (Qiagen) using an optimized blend of oligo-dT and random primers according to the manufacturer’s instructions. PCR was carried out on cDNA as previously described (14). Primer sequences are available as Supplementary Materials and Methods. To design a quantitative reverse transcription-PCR (qRT-PCR) assay, we used the Human ProbeLibrary system (Exiqon). Detailed procedure and primer sequences, as well as qRT-PCR experiments, are available as Supplementary Materials and Methods.

Protein extraction, Western blotting, and antibodies. Protein extraction and Western blotting procedure were carried out as reported elsewhere (14). Membranes were incubated with a primary antibody raised against the COOH terminus of the human CBX7 protein (Neosystem) for 60 min (at room temperature). To ascertain that equal amounts of protein were loaded, the Western blots were incubated with antibodies against the α-tubulin protein (Sigma). Membranes were then incubated with the horseradish peroxidase–conjugated secondary antibody (1:10,000) for 60 min (at room temperature) and the reaction was detected with a Western blotting detection system (enhanced chemiluminescence; GE Healthcare).

Histological analysis. The cell distribution of the CBX7 protein was assessed by immunostaining formalin-fixed, paraffin-embedded thyroid tumor blocks retrieved from the files of the Dipartimento di Scienze Biomorfolo giche e Funzionali at the Università di Napoli “Federico II” and selected to represent a wide range of thyroid neoplastic diseases. Briefly, xylene-de-waxed and alcohol-rehydrated paraffin sections were placed in Coplin jars filled with a 0.01 mol/L trisodium citrate solution and heated for 3 min in a conventional pressure cooker (14). After heating, slides were thoroughly rinsed in cool running water for 5 min. They were washed in TBS (pH 7.4) and then incubated overnight with the specific rabbit polyclonal primary antibody. Subsequently, tissue sections were stained with biotinylated anti-rabbit immunoglobulins and then with peroxidase-labeled streptavidin (Dako). The signal was developed by using diaminobenzidine chromogen as substrate. Incubations both omitting and preadsorbing the specific antibody were used as negative controls.

To ensure that we evaluated CBX7 expression only on technically adequate slides, we discarded slides that lacked a convincing internal control, namely labeling of stromal, endothelial, or lymphoid cell, shown to be positive in a preliminary normal tissue microarray analysis (data not shown). Based on these criteria, we scored paraffin-embedded stained slides from 20 cases of FTA, 30 cases of classic FTC, 6 cases of TCV PTC, 32 cases of FTC, 12 cases of PDC, and 12 cases of ATC. As controls, we selected areas of normal thyroid parenchyma from the lobe contralateral to the tumor in 20 surgical specimens of FTC. Individual cells were scored for the expression of CBX7 by quantitative analysis performed with a computerized analyzer system (Iba 2000, Kontron, Zeiss), as described previously (14), and tumors were subdivided into low expressors (<50% of positive cells) and high expressors (>50% of positive cells).

Thyroid fine-needle aspiration biopsies. The fine-needle aspiration biopsies (FNAB) were carried out at the Cytopathology Section of the Dipartimento di Scienze Biomorfolo giche e Funzionali (Università di Napoli “Federico II”) as described elsewhere (24, 25). To evaluate whether CBX7 gene expression analysis is also feasible on cytologic samples, we selected cell-block specimens obtained from 15 FNAB diagnosed as PTC and histologically confirmed. Five-micrometer paraffin-embedded sections were cut from each cell block and were examined for the presence of neoplastic cells by HE staining, before CBX7 immunocytochemistry and qRT-PCR had been carried out. Nonneoplastic cytologic specimens, used as controls, were obtained from the cell block corresponding to FNAB taken from goiter nodules. FNAB samples were processed for RNA extraction using the procedure described in a previous section.

Loss of heterozygosity analysis. We used several single nucleotide polymorphism (SNP) markers to evaluate the loss of heterozygosity (LOH) at the CBX7 locus on chromosome 22q13.1. We selected the SNP markers that showed high average heterozygosity levels to obtain the highest number of informative cases. Briefly, genomic DNA was PCR amplified in a region spanning about 200 bp around the SNP analyzed, then the purified PCR product was sequenced. We measured the height of the two peaks on the chromatogram and calculated the ratio of the two alleles in the matched tumor/normal samples: LOH was defined if the ratio in the carcinoma sample was <50%. SNPs and relative primer sequences are available as Supplementary Materials and Methods.

Plasmid constructs and cell low-growth-forming assay. CBX7 expression plasmid was constructed by cloning the human cDNA sequence in a PCRII TA Cloning vector (Invitrogen). The primers used were as follows: CBX7 forward 5'-ATGGAGCTGTCAGCCATC-3' and CBX7 reverse 5'-TCA-GAACCTTCCCACCTGC-3'. The inserted cDNA was then subcloned into the BamHI/XhoI sites of the mammalian expression vector pcDNA3.1 (Invitrogen). The expression of CBX7 was assessed by Western blotting.

Cells were plated at a density of 90% in 100-mm dishes, were transfected with 5 μg pcDNA3.1 or pCBX7 and supplemented with gentamicin (G418) 24 h later. Two weeks after the onset of drug selection, the cells were fixed and stained with crystal violet (0.1% crystal violet in 20% methanol).

Cell cycle analysis. Cells were harvested in PBS containing 2 mmol/L EDTA, washed once with PBS, and treated for 30 min in cold ethanol (70%). Cells were washed once in PBS and permeabilized with 0.2% TWEEN 20 and 1 mg/ml RNase A for 30 min, and washed and stained with 50 μg/ml propidium iodide. Stained cells were analyzed with a FACS Calibur (Becton Dickinson), and the data were analyzed using a Mod-It cell cycle analysis program.

Preparation of recombinant adenovirus and infection protocol. The recombinant adenovirus was constructed using the AdEasy Vector System (Quantum Biotechnologies). The cDNA fragment was inserted in the sense orientation into the NotI and Hind IIII sites of the pShuttle-CMV vector to
generate the recombinant pShuttle-CBX7-CMV construct. It was linearized and cotransformed through electroporation with pAdEasy-1, which carries the adenovirus genome, in BJ5183 electrocompetent cells. After homologous recombination, a recombinant AdEasy-CMV-CBX7 plasmid was generated (Ad-CBX7), which was then extracted and linearized. QBI-293A cells were transfected with different clones to produce different viral particles, and the infectivity of each clone was tested. Viral stocks were expanded in QBI-293A cells, which were harvested 36 to 40 h after infection and lysed. The virus titer of the 293 cells was determined. The adenovirus AdCMV-GFP (Quantum Biotechnologies) was used as control. Cells (5 × 10⁴) were

Figure 1. CBX7 expression in human thyroid carcinoma cell lines and neoplastic thyroid tissues. A, CBX7 gene expression by microarray analysis in human thyroid carcinoma cell lines versus human normal thyroid primary culture cells (NTPC). The average difference (Avg. Diff) is a quantitative relative indicator of a transcript expression level [Σ(PM-MM)] / pairs on average]. B, CBX7 gene expression analysis by RT-PCR in human thyroid carcinoma cell lines versus normal thyroid primary culture cells. β-Actin gene expression served as loading control. C, normal and neoplastic thyroid tissues analyzed for CBX7 protein expression by immunohistochemistry. D, CBX7 nuclear staining was intense in benign follicular epithelial cells of normal thyroid (1) and follicular adenoma (2), whereas it was weaker in malignant lesions (3), where normal thyroid and papillary carcinoma are adjacent. The decrease of CBX7 in neoplastic lesions was progressive going from well-differentiated cancer, such as minimally invasive follicular carcinoma (4) and “classic variant” papillary carcinomas (PTC; 5) to the “tall cell variant” of PTC (6), whose nuclei are magnified to better show lack of signal, to poorly differentiated (7) and anaplastic (8) carcinomas. The signal disappeared after incubation of the sample with antigen (9). Arrows with letters indicate the following sample features; P—, nuclei showing cytologic features of PTC negative for CBX7 expression; N—, normal thyroid adjacent to papillary cancer; L—, lymphocyte showing CBX7 expression and providing positive internal control.
seeded in a six-well plate. After 24 h, cells were infected at a multiplicity of infection (MOI) of 100 with Ad-CBX7 or Ad-GFP for 90 min using 500 μL of infection medium (DMEM supplemented with 2% fetal bovine serum) at 37°C in a 5% CO2 incubator. Pilot experiments with Ad-GFP were carried out to determine the optimal MOI for each cell line. At MOI 100, the cell lines became GFP positive without manifesting toxicity. Infected cells were harvested and counted daily in a hematocytometric chamber.

**Results**

**CBX7 gene expression is down-regulated in human thyroid carcinoma cell lines.** To look for genes potentially involved in the neoplastic transformation of the thyroid gland, we extracted RNAs from normal human thyroid primary cells and six human thyroid carcinoma cell lines (WRO cell line from FTC, TPC-1 and FB-2 cell lines from PTC, NPA cell line from a poorly differentiated PTC, and ARO and FRO cell lines from ATC) and hybridized them to U95Av2 Affymetrix oligonucleotide arrays (Affymetrix) containing 12,625 transcripts (14). We looked for genes whose expression was drastically (at least 10-fold) up- or down-regulated in all the six thyroid carcinoma cell lines versus normal thyroid primary cell culture, on the assumption that genes whose expression was altered in all carcinoma cell lines could be involved in thyroid cell transformation. Thus, genes that were decreased in all the carcinoma cell lines were considered candidate tumor suppressor genes.
genes. Among these genes, we decided to concentrate our studies on CBX7. The reasons of this choice were that CBX7 was one of the genes with the highest down-regulation in all the carcinoma cell lines, according to the data of the cDNA microarray analysis (Fig. 1A), and that previous studies suggested its involvement in the repression of gene transcription (13), and then, a possible tumor suppressor function. This result was confirmed by RT-PCR analysis in a large panel of thyroid carcinoma cell lines with normal thyroid primary culture cells as control (Fig. 1B).

The loss of CBX7 expression correlates with a more aggressive phenotype of thyroid carcinomas. Because thyroid carcinoma cell lines have a relative validity (26), we decided to carry out an immunohistochemical analysis of paraffin-embedded tissues using polyclonal antibodies raised against the carboxy-terminal region of the human CBX7 protein. As shown in Fig. 1C, all 20 samples of normal thyroid parenchyma expressed CBX7 at a high level, which coincides with the strong CBX7 staining in all follicles (Fig. 1D, subpanel 1). The intensity of nuclear labeling of epithelial thyroid cells in F1A (Fig. 1D, subpanel 2) and in goiters (not shown) was similar to that of normal thyroids and of the internal control represented by lymphoid cells. Conversely, CBX7 expression was reduced in malignant lesions, as it was evident when neoplastic and normal tissue areas were adjacent (Fig. 1D, subpanel 3). The percentage of low expressors was high in well-differentiated tumors, namely FTC (66%, 21 of 32 samples; Fig. 1C and D, subpanel 4) and PTC (57%, 17 of 30 samples; Fig. 1C and D, subpanel 5). It was even higher in the less differentiated tumors, namely PDC (83%, 10 of 12 samples; Fig. 1C and D, subpanel 7) and TCV PTC (83%, 5 of 6 samples; Fig. 1C and D, subpanel 6). In the latter, neoplastic cells were almost devoid of CBX7 expression, which sharply contrasted with the intense staining of the infiltrating lymphocytes and stromal cells. Similarly, CBX7 expression was completely lost in the neoplastic cells in all cases of ATC (100%, 12 of 12; Fig. 1C and D, subpanel 8). No staining was observed when normal thyroid gland samples were stained with antibodies preincubated with the peptide against which the antibodies were raised (Fig. 1D, subpanel 9) or in the absence of the primary antibodies (data not shown). Therefore, CBX7 was expressed in normal thyroid and in benign neoplastic lesions, decreased in well-differentiated carcinomas, and drastically reduced in aggressive thyroid tumors.

Analysis of CBX7 expression in normal and neoplastic thyroid tissues by RT-PCR and quantitative real-time PCR. We also evaluated CBX7 expression by RT-PCR in a panel of matched normal/tumor tissues. The results confirmed the immunohistochemical data. In fact, there was an amplified band corresponding to CBX7 in normal thyroid tissues (Fig. 2A). The amplified band disappeared in PTC samples (Fig. 2A, top) and almost disappeared in ATC (Fig. 2A, bottom). Quantitative qRT-PCR analysis of a large number of human thyroid carcinoma samples of different histotypes confirmed a correlation between the reduction of CBX7 expression and a more malignant phenotype of thyroid neoplasias. In fact, as reported in Fig. 2B, there was a negative fold change in CBX7 expression from −21.1 to −13 (average −4.8) in the PTC samples versus the normal counterpart tissues. The reduction was even more pronounced in the ATC samples, with a fold change ranging from −10.8 to −24.5 (average −14.6). These data are well correlated with the immunohistochemical data and suggest that CBX7 expression is controlled at the transcriptional level.

Analysis of human thyroid FNAB. FNAB has become an integral part of the preoperative evaluation of thyroid nodules. To evaluate whether CBX7 gene expression analysis is also feasible preoperatively, immunocytocchemistry and qRT-PCR were carried out on cell block specimens obtained from 15 FNAB diagnosed as PTC and histologically confirmed. In 8 cases of 15, CBX7 expression was lower in PTC cell blocks than in specimen from thyroid goiter, as evaluated by immunocytochemistry and qRT-PCR (Fig. 2C). This percentage was quite similar to that obtained by analyzing paraffin-embedded tissues diagnosed as PTC by immunohistochemistry.

LOH at CBX7 locus. In some types of cancers, LOH of tumor suppressor genes at region 22q is believed to be a key step in carcinogenesis (27, 28). We therefore used several SNP markers to evaluate LOH at the CBX7 locus on chromosome 22q13.1 in 77 cases of thyroid carcinomas of different histotypes. As shown in Table 1, LOH at the CBX7 locus occurred in 36.8% of the informative PTC (7 of 19 cases) and in 68.7% (11 of 16 cases) of informative ATC. No LOH was observed in FTA (0 of 6 cases).

**Table 1. LOH frequency statistics at the CBX7 locus (22q13.1) by SNP sequencing method**

<table>
<thead>
<tr>
<th>Histotype</th>
<th>Cases (Inf)*</th>
<th>LOH 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Folicular adenoma</td>
<td>10 (6)</td>
<td>0% (0/6)</td>
</tr>
<tr>
<td>Folicular carcinoma</td>
<td>5 (3)</td>
<td>66.6% (2/3)</td>
</tr>
<tr>
<td>Papillary carcinoma—classic variant</td>
<td>36 (19)</td>
<td>36.8% (7/19)</td>
</tr>
<tr>
<td>Papillary carcinoma—tall cell variant</td>
<td>6 (4)</td>
<td>75% (3/4)</td>
</tr>
<tr>
<td>Anaplastic carcinoma</td>
<td>20 (16)</td>
<td>68.7% (11/16)</td>
</tr>
</tbody>
</table>

*Informative cases (Inf) are samples showing SNP heterozygosity corresponding to two peaks (two alleles) on the sequencing chromatogram.

1 The LOH frequency is equal to the ratio between allelic loss and informative cases.
undergo morphologic changes and lose the thyroid differentiation markers, they are unable to grow in soft agar and to induce tumors after injection into athymic mice (21, 23). Therefore, the analysis of rat thyroid cells transformed in vitro also confirms that the loss of CBX7 expression is associated with the expression of a highly malignant phenotype.

In addition, we used qRT-PCR analysis to evaluate CBX7 expression in thyroid neoplasias developing in transgenic animal lines expressing some oncogenes under the transcriptional control of the thyroglobulin promoter. Transgenic mice carrying TRK and RET/PTC3 oncogenes develop PTC (29, 30), whereas N-ras mice develop thyroid follicular neoplasms that undergo dedifferentiation, predominantly FTC (31). ATCs were obtained from mice carrying SV40 large T antigen (32). As shown in Fig. 3B, CBX7 expression was much lower in ATC from large T SV40 transgenic mice compared with mouse normal thyroid tissue. CBX7 mRNA expression was significantly, albeit not greatly, reduced in PTC from TRK and RET/PTC3 mice. CBX7 expression was also reduced in the FTC from N-ras mice.

**Restoration of CBX7 gene expression inhibits the growth of thyroid carcinoma cell lines.** To determine whether the loss of CBX7 gene expression affects thyroid carcinogenesis, we evaluated the growth rate of three thyroid carcinoma cell lines in which CBX7 expression had been restored. To this aim, we carried out a colony-forming assay with three human thyroid carcinoma cell lines (ARO, NPA, TPC-1) after transfection with the vector carrying the CBX7 gene or the backbone vector. As shown in Fig. 4A, cells transfected with the CBX7 gene grew at a significantly slower rate than cells transfected with the backbone vector did.

Moreover, we have analyzed the growth potential of CBX7 stably expressing ARO cells by a 2,3-bis[2-methoxy-4-nitro-5-sulphonyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay at different times after plating. As shown in Fig. 4B, the ARO cells expressing the CBX7 gene grew at a significantly slower rate from TRK and RET/PTC3 mice. CBX7 expression was also reduced in the FTC from N-ras mice.

**Figure 3. CBX7 expression in experimental models of thyroid carcinogenesis.** A, CBX7 expression by RT-PCR in rat thyroid cells transformed by several oncogenes. GAPDH gene expression was evaluated as control to normalize the amount of the used RNAs. B, CBX7 expression by qRT-PCR in thyroid carcinomas developing in transgenic mice expressing RET-PTC-3, TRK, N-ras, and large T SV40 oncogenes. The fold change indicates the relative change in expression levels between tumor samples and normal samples, assuming that the value of normal sample is equal to 1.
compared with the untransfected and backbone vector-transfected ARO cells.

We then investigated the cell cycle phase distribution of these CBX7 stably transfected ARO cells through flow cytometric analysis. As shown in Fig. 4C, ARO cells expressing CBX7 had a significant increase in the G1-phase population (ARO CBX7-1, 66%; ARO CBX7-8, 64%), compared with empty vector–transfected cells (ARO HA-3, 49%; ARO HA-4, 51%) and parental cells (ARO, 48%). Therefore, these results suggest that CBX7 negatively regulates thyroid carcinoma cell proliferation.

**Generation of an adenovirus carrying the CBX7 gene.** We generated a replication-defective adenovirus carrying the CBX7 gene in the sense (Ad-CBX7) orientation. We then infected thyroid carcinoma cell lines with the Ad-CBX7 virus and measured protein levels in cell lysates collected at different time points after adenovirus infection. No CBX7 protein was detected in carcinoma cells infected with the control virus (Ad-GFP). We then constructed growth curves of cells infected with Ad-CBX7 and control adenovirus (Ad-GFP). As shown in Fig. 4D, cell growth rate was significantly lower in ARO and NPA cell lines infected with Ad-CBX7 than in the same cells infected with the control virus. The percentage of growth inhibition 5 days after infection was 38.5% in ARO cells and 54% in NPA cells.

**Expression of CDKN2A/p16 in human thyroid tumor samples.** It has been reported that a target of CBX7 would be the CDKN2A/p16 tumor suppressor gene (11, 12, 33), suggesting that overexpression of CBX7 could enable cells to evade oncogene-induced senescence; we then evaluated CDKN2A/p16 expression in

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**Figure 4.** Effect of CBX7 restoration on thyroid carcinoma cell lines. A, colony-forming assay with CBX7 transfection in several thyroid carcinoma cell lines (ARO, NPA, and TPC-1). B, growth curve of ARO cells and ARO cells stably carrying a vector expressing CBX7 (ARO CBX7-1 and ARO CBX7-8) or the backbone vector (ARO HA-3 and ARO HA-4). The relative number of viable cells was determined by the XTT assay. Absorbance was read at 450 nm and the data are the mean of triplicates. C, CBX7 expression affects the cell cycle distribution of ARO cells as assessed by flow cytometric analysis. The expression of the CBX7 protein in ARO CBX7-1 and ARO CBX7-8 was confirmed by Western blot. D, top, inhibitory effects of Ad-CBX7 infection on the growth of human thyroid carcinoma cell lines. Representative curves of three independent experiments are reported. Bottom, CBX7 expression by Western blot analysis. Blot against α-tubulin is to show an equal protein loading.
Discussion

Microarray studies are widely used to define diagnostic and prognostic signatures in cancers and they have led to the identification of a large list of carcinoma-regulated genes also in thyroid cancers (37). Just within the context of a microarray study, we found that the CBX7 gene was drastically down-regulated in all thyroid carcinoma–derived cell lines analyzed.

The results of this study show that CBX7 expression decreased with malignancy grade and neoplasia stage. In fact, CBX7 expression was comparable with normal thyroid tissue in FTA, which is benign; slightly reduced in PTC displaying the classic histotype; and drastically reduced, and in most cases absent, in FTC, TCV PTC, PDC, and ATC. Our finding of a decrease in CBX7 levels in relation to malignancy was supported by our model of rat thyroid cells transformed by several oncogenes and in transgenic mice carrying thyroglobulin promoter-driven oncogenes. CBX7 expression was absent in rat thyroid cells that show a highly malignant phenotype and in ATC that develop in large T SV40 transgenic mice. Differently, CBX7 expression was retained, albeit at a low level, in transformed rat thyroid cells that are not yet tumorigenic and in PTC developing in RET/PTC and TRK mice. Therefore, our data indicate that loss of CBX7 expression correlates with a more aggressive phenotype of thyroid carcinomas and, likely, with a worse prognosis. Interestingly, a recent article concerning the cytogenetics of Chernobyl thyroid tumors identified a correlation between the deletion of the chromosomal region 22q13.1, where the CBX7 gene is located, and a worse prognosis (38). Our preliminary finding shows a correlation between low CBX7 expression and reduced survival in colon carcinoma.5 Moreover, the association between lack of CBX7 expression and a more aggressive histotype seems to apply also to breast, ovary, and lung carcinomas.6

To determine whether CBX7 may contribute to thyroid carcinogenesis, we restored the CBX7 function in human thyroid cancer cell lines and examined cell growth rate. Restoration of CBX7 expression reduced cell growth rate, indicating that the loss of CBX7 expression may play a role in thyroid carcinogenesis.

In the attempt to unravel the mechanism underlying the loss of CBX7 gene expression in malignant thyroid neoplasias, we analyzed LOH at the CBX7 locus (22q13.1). We detected LOH in 36.8% and 68.7% of the PTC and ATC, respectively, but not in FTA. However, no mutations were found in thyroid carcinomas; moreover, no hypermethylation status was observed in ATC (data not shown), which are practically devoid of CBX7 expression. Therefore, we suggest that other epigenetic mechanisms associated with an allelic loss might account for the reduced CBX7 expression in thyroid carcinomas. Consistent with this hypothesis, our preliminary data indicate that the HMGA1 proteins, also overexpressed in thyroid carcinomas, can be directly down-regulated by the CBX7 expression.

The data reported here seem to propose CBX7 as a tumor suppressor gene. However, recent publications (11, 12, 33) seem to attribute oncogenic functions to CBX7. In fact, it has been shown, by the generation of transgenic mice overexpressing CBX7, that CBX7 can initiate T-cell lymphomagenesis and cooperate with c-Myc to produce highly aggressive B-cell lymphomas (33). Moreover, it has been also shown that CBX7 expression facilitates the survival of the mouse embryonic fibroblasts (11), consistently with a CBX7 oncogenic role. These contrasting results are, in our opinion, not mutually exclusive at all. The cellular context can account for the opposite functions attributed to CBX7. We retain that this can occur with a certain frequency with chromatin proteins: the cellular partners, which may vary from cell to cell, can modify the action exerted by them. This hypothesis is supported by the studies on the HMGA1 proteins that are overexpressed in most of the malignant tumors, and in vitro and in vivo studies showed the oncogenic activity of overexpressed HMGA proteins (40–42). However, the phenotype of the null mice revealed that the HMGA1 gene plays a hitherto unsuspected tumor suppressor role because they developed, even at the heterozygous state, B-cell lymphomas and myeloid malignancies (43). There are also evidence that some E2F family members are able to act as both oncogene and tumor suppressor gene depending on the context (44). In fact, E2F1 expression can either promote or inhibit tumorigenesis depending on the nature of the other oncogenic mutations that are present (44). The dual role of the CBX7 gene as an oncogene or a tumor suppressor is also suggested by the loss of CBX7 expression in pediatric ependymomas (45), and also by our recent data showing a correlation between CBX7 overexpression and a high malignant

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phenotype in head and neck tumors, although an inverse correlation has been found in ovary, lung, and colon carcinomas. Moreover, mouse embryonic fibroblasts (MEF) null for CBX7 (generated in our laboratory) have a higher proliferation rate than the wild-type MEFs, whereas the CBX7+/− MEFs show an intermediate behavior, suggesting a negative role of the CBX7 protein in the growth of these cells (Supplementary Fig. S1).

In conclusion, our data indicate that a reduced CBX7 gene expression is associated with a malignant phenotype of thyroid neoplasias and suggest that the loss of CBX7 could contribute to thyroid cancer progression.

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

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References
19. Berlingieri MT, Santoro M, Battaglia C, Greco M, Fusco A. The Adenovirus E1A gene blocks the differentiation of a thyroid epithelial cell line, however the neoplastic phenotype is achieved only after cooperation with other oncogenes. Oncogene 1993;8:249–55.

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