Evidence of oncogene-induced senescence in thyroid carcinogenesis

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Abstract

Oncogene-induced senescence (OIS) is a growth arrest triggered by the enforced expression of cancer-promoting genes and acts as a barrier against malignant transformation in vivo. In this study, by a combination of in vitro and in vivo approaches, we investigate the role of OIS in tumours originating from the thyroid epithelium. We found that expression of different thyroid tumour-associated oncogenes in primary human thyrocytes triggers senescence, as demonstrated by the presence of OIS hallmarks: changes in cell morphology, accumulation of SA-β-Gal and senescence-associated heterochromatic foci, and upregulation of transcription of the cyclin-dependent kinase inhibitors p16INK4a and p21CIP1. Furthermore, immunohistochemical analysis of a panel of thyroid tumours characterised by different aggressiveness showed that the expression of OIS markers such as p16INK4a, p21CIP1 and IGFBP7 is upregulated at early stages, and lost during thyroid tumour progression. Taken together, our results suggest a role of OIS in thyroid carcinogenesis.

Introduction

Thyroid tumours represent the most common endocrine malignancy; the majority originate from thyroid follicular cells and include differentiated follicular thyroid carcinoma (FTC), papillary thyroid carcinoma (PTC), poorly differentiated thyroid carcinomas (PDTC) and undifferentiated anaplastic thyroid carcinomas (ATC) (Sheils 2005).

PTC accounts for ~80% of all thyroid cancers; generally it is a slow-growing tumour type with a good prognosis, although rare, aggressive forms with local invasion or distant metastasis can occur. In addition to classical PTC, also known as not otherwise specified (NOS), several histological variants, differing in morphological pattern as well as in prognosis, have been described, including papillary thyroid microcarcinomas (PTMC), solid variant (SV), PDTC and tall cell variant (TCV) (Sheils 2005). PTMC is defined as a tumour measuring ≤1 cm in diameter and is considered the early stage of PTC. It has an indolent course and very favourable prognosis despite the presence of multifocality within the thyroid and the synchronous nodal loco-regional spread (Sakorafas et al. 2005). The molecular mechanisms underlying PTC pathogenesis have been partially elucidated. PTCs are associated with genetic alterations leading to the activation of the ERK1/2 signal pathway, including point mutations of the BRAF and RAS genes, and rearrangements of RET and NTRK1 tyrosine kinase receptors, producing RET/PTC and TRK oncogenes respectively (Greco et al. 2009). Of note, BRAF mutations have been reported in a consistent fraction of PTMC (Lupi et al. 2007), thus supporting the notion that it represents an earlier stage of disease which eventually evolves into PTC. Nevertheless, the molecular mechanisms driving the evolution of PTMC...
in overt carcinoma or, vice versa, keeping PTMC proliferation under control, as well as the mechanisms responsible for the most aggressive forms of PTC, remain to be elucidated.

When expressed in primary cells, activated oncogenes can block cellular proliferation by inducing senescence or apoptosis (Campisi 2005, Mooi & Peep er 2006). Both mechanisms are thought to play important roles in suppressing tumourigenesis in vivo, as they prevent the proliferation of cells at risk of neoplastic transformation (Michaloglou et al. 2005). The factors that direct the choice of cancer cells into one or the other outcome have remained unclear (D’Adda di Fagagna 2008); most likely they are related to the oncogenic trigger and to the balance between proapoptotic and antiapoptotic factors.

Oncogene-induced senescence (OIS) was first identified in vitro and describes a stable cell-cycle arrest that is triggered by the aberrant proliferative signals of oncogenes (Serrano et al. 1997). Senescent cells are characterised by a flat and large morphology with vacuoles (Denoyelle et al. 2006), an increase in senescence-associated β-galactosidase activity (SA-β-Gal; Dimri et al. 1995), and alterations in chromatin structure known as senescence-associated heterochromatic foci (SAHF), which may repress the expression of proliferation-promoting genes (Narita et al. 2003, Adams 2007). Moreover, senescent cells undergo dramatic changes in gene expression pattern, including, activation of p53, increased levels of the cyclin-dependent kinase inhibitors, p16INK4a and p21CIP1 (Beausejour et al. 2003, Lowe et al. 2004) and secretion of multiple factors such as extracellular proteases, matrix components, growth factors, pro-inflammatory cytokines and chemokines (Kortlever et al. 2006, A costa et al. 2008, Kuilman et al. 2008, Wajapeyee et al. 2008).

OIS is a mechanism of increasing importance in tumours (Kuilman et al. 2010), as several reports have demonstrated that it occurs in several pre-cancerous tissues from human and mice, and acts as a barrier to invasiveness and tumourigenesis. The first direct evidence of cellular senescence in a growth arrested human neoplasm was reported in melanocytic nevi, premalignant lesions frequently carrying the \( BRAF^V600E \) mutation which rarely progress to melanoma (Michaloglou et al. 2005). The blunted proliferative activity in nevi was associated with features of cellular senescence, including SA-β-Gal activity and elevated levels of p16\(^{INK4a} \). On the contrary, in melanoma, which is characterised by accelerated proliferation, cell senescence was extinguished. Later on, senescence markers were identified in human dermal neurofibromas, murine lung adenomas, human and murine prostatic adenomas, murine pancreatic intraductal neoplasias and murine lymphomas (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005, Courtois-Cox et al. 2006, Dankert et al. 2007, Ha et al. 2007). The increasing evidence of OIS involvement in human cancer suggests the possibility of new therapeutic approaches based on the functional restoration of OIS in tumour cells.

In this study, we investigate the occurrence of OIS in thyroid carcinogenesis with in vitro and in vivo studies. We found that PTC-associated oncogenes trigger OIS in primary human thyrocytes, as demonstrated by changes in cell morphology and the presence of specific OIS markers. To assess whether this mechanism represents a barrier to thyroid cancer in vivo, we performed immunohistochemical analysis in a panel of thyroid tumours characterised by different aggressiveness. OIS markers were found to be upregulated in PTMCs, whereas they were progressively lost in PTCs and ATC. Overall, our data support a role for OIS in thyroid carcinogenesis.

Materials and methods

Cell culture

Normal thyroid samples were obtained from patients undergoing surgery at IRCCS Foundation Istituto Nazionale dei Tumori (Milan, Italy). All patients gave their written informed consent, and the study was approved by the independent ethics committee of IRCCS Foundation Istituto Nazionale dei Tumori. Primary thyrocyte cultures were established and maintained in nutrient mixture Ham’s F12 medium (custom made by Invitrogen) containing 5% calf serum and bovine hypothalamus and pituitary extracts, as described previously (Curcio et al. 1994). Thyrocytes expressing \( RET/PTC1 \) oncogene, obtained from infection of primary thyrocytes with \( RET/PTC1 \) retroviral vector, have been previously described (Borrello et al. 2005). HEK293T cells were maintained in DMEM F9 medium supplemented with 10% FCS.

Lentivirus production and transduction

HEK293T cells \((4 \times 10^5)\) were transfected with 8 µg of \( BRAF^V600E \) lentiviral construct (HIV-CS-CG-Blast-BRAF\(^{V600E}\)) and 3 µg of each of the helper plasmids pMDLgIpRRE, pHCMV-G and pRSVrev by calcium phosphate in complete medium containing 25 µM chloroquine. After 6–12 h of incubation cells were washed and refed with complete medium. Supernatant containing lentiviral particles was collected and frozen.
24 h after transfection, and subsequently added to thyrocytes for 6 h in the presence of 0.8 μg/ml polybrene (Sigma–Aldrich), followed by blasticidin selection (5 μg/ml) after 24 h. Three days later, cells were harvested and plated at different densities for further analysis. The efficiency of transduction was monitored in parallel infections using a virus expressing enhanced green fluorescent protein (eGFP).

**p16INK4a shRNA silencing**

p16INK4a knockdown constructs were created in KH1 lentiviral vectors, carrying the GFP reporter gene, with the following sense shRNA sequences: shp16.5 (TGCCCCCGGGGGAGACCCAAC) and shp16.13 (GCCGACCGACCCTCTCTCA) and used for thyrocytes transduction. Transduction efficiency was monitored by GFP expression. Six days later, cells were re-transduced with lentivirus encoding BRAFV600E (HIV-CSCG-BRAFV600E) and maintained under blasticidin selection for an additional 6 days.

**Nucleofection**

Human primary thyrocytes (1 × 10^6) were resuspended in 110 μl human keratinocyte nucleofector solution (Amaxa, Lonza AG Basel, Switzerland) together with 1 μg of plasmid DNA, transferred to the cuvette and nucleofected using a Amaza Nucleofector II device (programme T003). Immediately after nucleofection, cells were resuspended in pre-heated complete culture medium and then seeded at different densities for further analysis. Cells were refed 24 h later and subjected to G418 (400 μg/ml) selection. Plasmid vectors carrying TRK-T3 and H-RASG12V oncogenes have been previously described (Pulciani et al. 1982, Greco et al. 1995).

**BrdU incorporation**

Cells were labelled with 10 μM BrdU for 3 h, harvested and fixed in 75% ethanol for 30 min. After washing with PBS, cells were treated with RNase A (500 μg/ml) for 30 min, permeabilised with 5 M HCl/0.5% Triton for 20 min at room temperature, neutralised with 1 M Tris, and washed in 0.5% Tween/PBS. Incubation with primary anti-BrdU antibody (30 min at room temperature) and secondary FITC-conjugated antibodies (30 min at room temperature; Dako, Seattle, WA, USA) was followed by staining with propidium iodine (20 μg/ml) at 37 °C for 30 min. Samples were analysed by flow cytometry (Becton Dickinson, San Jose, CA, USA).

SA-β-Gal assay

Adherent cells were analysed for SA-β-Gal production using a Senescence-beta-Galactosidase Staining Kit (Cell Signaling, Danvers, MA, USA) following the manufacturer’s instructions. For each sample at least 200 cells were scored.

**SAHFs formation**

For the analysis of SAHFs, transfected/transduced cells growing on glass cover slips were fixed in 4% paraformaldehyde/2% sucrose, permeabilised with 0.1% Triton, and stained with Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen). Stained nuclei were observed under a fluorescent microscope (Nikon Eclipse E1000). For each sample at least 100 cells were scored.

**Western blotting analysis**

Proteins were extracted in RIPA modified buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% NonidetP-40) supplemented with Complete Mini EDTA-free protease Inhibitor Cocktail (Roche), 1 mM Na_2VO_4 and 1 mM PMSF. Protein samples were quantified by Bradford’s assay with BIO-RAD Protein Assay (Bio-Rad). Supernatant obtained by incubating cells in serum-free medium for 24 h were concentrated by centrifugation at 3200 g using AgilentSpin Concentrators (Agilent Technologies, Inc., Wilmington, DE, USA) and normalised to cell number. Protein and supernatant samples were boiled in NuPAGE LDS sample buffer (Invitrogen) and separated on 4–12 or 10% NuPAGE Novex Gel (Invitrogen) with MOPS or MES running buffer respectively. Proteins were transferred onto nitrocellulose filters and immunoblotted with the following primary antibodies to: p16INK4a (BD Becton Dickinson, Franklin Lakes, NJ, USA); p21CIP1, BRAF (F7), RET (C-19), TRK (C-14), IGFBP7 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA); p53 (YLEM); phospho-ERK1/2 (p-ERK1/2), ERK1/2, β-actin (Sigma–Aldrich); RAS (Ab3; Calbiochem, San Diego, CA, USA); HMGA2 (kindly provided from G Manniello, University of Trieste). The immunoreactive bands were visualised using HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

**Real-time RT-PCR**

RNA was isolated with TRIzol reagent (Invitrogen) from cells collected at days 1 and 7 postinfection and purified with RNeasy purification kit (Qiagen). Reverse transcription was performed with Superscript
Reverse Transcriptase III following the manufacturer’s instructions (Invitrogen). For each sample, 20 ng of retro-transcribed RNA were amplified in PCRs carried out in triplicate on an ABI PRISM 7900 using TaqMan gene expression assays (Applied Biosystem, Foster City, CA, USA). Hs00266026_A1 was used for IGFBP7 expression; human HPRT (HPRT-Hs99999909_A1) was used as housekeeping gene for normalisation among samples. Data analysis was performed by the Sequence Detection System (SDS) 2.2.2 software (Applied Biosystems, Foster City, CA, USA).

**Tumour samples and immunohistochemistry**

The thyroid tumour samples which were analysed were selected by reviewing a larger tumour series available at the Department of Pathology from patients operated on at IRCCS Foundation Istituto Nazionale dei Tumori (Milan, Italy) from 2002 to 2009. Tumours were classified according to the histopathological typing of the World Health Organization (DeLellis et al. 2004). Tumour collection included: PTMC (six cases); PTC NOS (six cases); PTC SV (one case; Nikiforov et al. 2001); PDTC (two cases); ATC (one case). Clinical pathological features are reported in Table 1. All patients gave their written informed consent, and the study was approved by the independent ethics committee of IRCCS Foundation Istituto Nazionale dei Tumori.

The immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue sections of 2 μm thickness. Antigen retrieval was performed by 1 mM citrate buffer (pH 6) or 0.25 mM EDTA (pH 8) in an autoclave at 95 °C for 6–15 min. Incubation with primary antibodies was performed overnight at 4 °C for anti-p21 CIP1 and anti-IGFBP7 (Santa Cruz Biotechnology); for 1 h at room temperature for anti-human Ki-67 Antigen (Mib1; DakoCytomation). Sections were then incubated for 30 min with biotinylated anti-goat, developed using 3,3′-diaminobenzidine (DakoCytomation, Glostrup, Denmark) as chromogen and finally counterstained with hematoxylin.

p16INK4a staining was performed by CINtec Histologic Kit (mtm Laboratories AG, Heidelberg, Germany) following the manufacturer’s instructions.

The intensity of staining for IGFBP7 was scored from (−) negative to (3+) strong. The percentage of cells immunostained for p16INK4a and p21CIP1 ranged from (−) <10% to (3+) 71–100%. Ki-67 staining was scored as very low (vL, 1–5% positive cells), low (L, 6–10% positive cells), medium (M, 11–30% positive cells) and high (H, ≥31% positive cells).

**Results**

**PTC-associated oncogenes induce senescence in primary human thyrocytes**

We investigated the capability of PTC-associated oncogenes, namely BRAFV600E, RET/PTC1, TRK-T3 and H-RASG12V, to trigger OIS in human primary thyrocytes.

**Table 1** Clinical–pathological information of thyroid tumour case collection

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Tumour type</th>
<th>Genetic lesion</th>
<th>ITS/LNM</th>
<th>Length of follow-up (months)</th>
<th>Clinical outcome</th>
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<tr>
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<td>ITS, LNM</td>
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<td>NED</td>
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<tr>
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<td>ITS, LNM</td>
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<td>NED</td>
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<tr>
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<td>–</td>
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<tr>
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<td>–</td>
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<td>BRAFV600E</td>
<td>LNM</td>
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<td>Progression</td>
</tr>
<tr>
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<tr>
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<td>UN</td>
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<td>UN</td>
<td>ITS</td>
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</table>

PTC, papillary thyroid carcinoma; PTMC, papillary thyroid microcarcinoma; NOS, not otherwise specified; SV, solid variant; PDTC, poorly differentiated thyroid carcinoma; ATC, anaplastic thyroid carcinoma; UN, unknown (indicate case negative for BRAFV600E mutations, and RET or TRK rearrangement); ND, not determined; ITS, intrathyroidal spreading; LNM, lymph node metastasis; NED, no evidence of disease.
We first performed transduction of primary human thyrocytes with a lentiviral vector carrying $\text{BRAF}^{\text{V600E}}$, the most frequent oncogene in PTC; as control, thyrocytes infected with lentivirus encoding only the blasticidin resistance gene were used. After selection with blasticidin, cells were monitored for morphology, proliferation and the presence of senescence-specific markers. As early as 4 days after infection, $\text{BRAF}^{\text{V600E}}$-transduced thyrocytes displayed a spindle-shaped morphology compared with cells transduced with the empty vector (Fig. 1A). Concomitantly, a marked growth arrest was observed, whereas control cells continued to proliferate. Thus, as measured 7 days after infection, thyrocytes transduced with $\text{BRAF}^{\text{V600E}}$ exhibited a dramatic reduction of S phase (0.47% of cells incorporated BrdU, vs 18.7% for control cells), and they accumulated preferentially in G1 phase (65% in cells expressing $\text{BRAF}^{\text{V600E}}$ vs 51% for control cells; Fig. 1B).

$\text{BRAF}^{\text{V600E}}$-transduced thyrocytes were next analysed for the presence of two well-known OIS markers: SA-β-Gal activity and SAHFs. A high portion of thyrocytes expressing $\text{BRAF}^{\text{V600E}}$ exhibited SA-β-Gal activity (85%) and SAHFs (80%), whereas in thyrocytes transfectected with the empty vector SA-β-Gal activity and SAHFs were detected in 8 and 3% of cells respectively (Fig. 1C).

The expression of $\text{BRAF}^{\text{V600E}}$, of its downstream effector ERK1/2, and of proteins involved in OIS such as p16$^{\text{INK4a}}$, p21$^{\text{CIP1}}$, p53 and HMGA2, a structural component of SAHFs (Narita et al. 2006), was investigated at days 7 and 14 postinfection in oncogene and empty vector-transduced thyrocytes (Fig. 1D). $\text{BRAF}^{\text{V600E}}$ was expressed to similar levels at both time points, in large excess with respect to endogenous BRAF. Concomitantly, an increase of ERK1/2 phosphorylation, with respect to control, was detected at day 7, and persisted at day 14. Importantly, $\text{BRAF}^{\text{V600E}}$ induced expression of p16$^{\text{INK4a}}$, further increased at day 14, whereas p53 and p21$^{\text{CIP1}}$ levels increased slightly only at the later time point. Moreover, elevated expression of HMGA2 protein was
maintained up to 14 days after transduction in the BRAF<sup>V600E</sup>-expressing cells, thus corroborating the heterochromatin changes detected by DAPI staining.

To rule out the possibility that OIS observed in thyrocytes could be related to the abnormal BRAF<sup>V600E</sup> expression levels obtained by lentiviral transduction, we performed nucleofection of thyrocytes, thus achieving a low level of expression of the oncoprotein (two-three times more abundant than endogenous BRAF, data not shown). A significant number of SA-β-Gal-positive (50%) and SAHFs-bearing (30%) cells with respect to control (10 and 4%, respectively) were detected (data not shown).

Taken together, these results indicate that the BRAF<sup>V600E</sup> oncogene induces senescence in human thyrocytes, and this is associated with the activation of the ERK1/2 pathway, p16<sup>INK4a</sup>, p21<sup>CIP1</sup> and p53 signal transduction cascades.

To investigate the role of p16<sup>INK4a</sup> in BRAF<sup>V600E</sup>-mediated senescence in thyrocytes, we suppressed the expression of p16<sup>INK4a</sup> using two independent lentiviral shRNA vectors (#5 and #13). Six days after infection, cells were re-transduced with a lentiviral vector encoding BRAF<sup>V600E</sup>, selected for expression of the oncogene, and analysed 7 days later (Fig. 2). Both shRNAs could silence p16<sup>INK4a</sup> expression. However, p16<sup>INK4a</sup> depletion did not affect the level of ERK1/2 phosphorylation induced by BRAF<sup>V600E</sup> nor basal p21<sup>CIP1</sup> levels (Fig. 2A). Similarly, inhibition of p16<sup>INK4a</sup> expression did not rescue the growth arrest caused by BRAF<sup>V600E</sup> (Fig. 2B). The percentage of p16<sup>INK4a</sup>-null BRAF<sup>V600E</sup> thyrocytes positive for SA-β-Gal activity was similar to BRAF<sup>V600E</sup> cells, whereas the percentage of p16<sup>INK4a</sup>-null BRAF<sup>V600E</sup> thyrocytes with SAHFs was reduced by ~40% compared with BRAF<sup>V600E</sup> cells (Table in Fig. 2C). This data indicated p16<sup>INK4a</sup> is not necessary for the execution of BRAF<sup>V600E</sup>-induced senescence in thyrocytes.

Figure 2 Effect of p16<sup>INK4a</sup> silencing on BRAF<sup>V600E</sup>-induced senescence in human primary thyrocytes. Cells were transduced with two non-overlapping shRNA against p16<sup>INK4a</sup> (5 and 13) upon infection with BRAF<sup>V600E</sup> encoding lentivirus. The analysis of OIS markers was performed at 14 days after infection with the two shp16<sup>INK4a</sup> constructs. (A) Expression of the indicated proteins was determined by western blot analysis. β-Actin was used as a loading control. (B) The percentage of cells for BrdU incorporation is shown. (C) The percentage of cells positive for each indicated markers is shown in the table. V, empty vector; B, BRAF<sup>V600E</sup>.

Figure 3 Detection of IGFBP7 expression in BRAF<sup>V600E</sup>-transduced human primary thyrocytes. The expression of IGFBP7 in primary thyrocytes infected with control lentivirus or lentivirus-expressing BRAF<sup>V600E</sup> was monitored by western blot analysis 7 days after infection (A) and by real-time RT-PCR analysis 1 and 7 days after infection (B). V, control vector; B, BRAF<sup>V600E</sup>; RQ, relative quantity of IGFBP7 mRNA normalised for HPRT housekeeping gene expression.
IGFBP7 is a secreted protein recently proposed as a mediator of BRAF$^{\text{V600E}}$-induced senescence in melanocytes (Wajapeyee et al. 2008); this role, however, has been questioned by other authors (Scurr et al. 2010). Interestingly, our recent results implicate IGFBP7 as a tumour suppressor protein in thyroid tumours: its expression is frequently lost in FTC and PTC, and its restoration modulates the transformed features of PTC-derived tumour cells (Vizioli et al. 2010). To investigate the possible involvement of IGFBP7 in BRAF$^{\text{V600E}}$-induced thyrocytes senescence we analysed the expression of soluble IGFBP7 in thyrocytes 7 days after BRAF$^{\text{V600E}}$ transduction. BRAF$^{\text{V600E}}$-infected cells showed reduced levels of secreted IGFBP7 protein compared with the control infected cells (Fig. 3A). To further characterise such a reduction we performed real-time RT-PCR (Fig. 3B). Also in this case, we observed a decrease in IGFBP7 mRNA expression in senescent cells (day 7). Of note, such a reduction was detectable as early as 1 day after infection.

Next, to investigate the effect of RET/PTC oncogenes in primary thyrocytes we used cells transduced with a retrovirus encoding the RET/PTC1 oncogene produced in the course of a previous study, and shown to display an increased growth rate compared with control uninfected thyrocytes, as determined in analyses performed between passages 3 and 7 after transduction (Borrello et al. 2005). Later on, however, by extending the number of passages in culture with respect to the previous study, we noted that RET/PTC1-infected thyrocytes, in contrast to control cells, ceased proliferating and showed several morphological abnormalities, which were not further investigated at that time. In the course of the present work, we re-evaluated the previous observation, taking advantage of frozen stocks of RET/PTC1-transduced thyrocytes and relative controls. Here again, 10–12 passages after infection, RET/PTC1-expressing cells ceased proliferating and displayed morphological features of senescence, such as extensive vacuolisation. Moreover, SA-β-Gal and SAHFs were found in 80 and 70%, respectively, of cells infected with RET/PTC1, but only in 10 and 2%, respectively, of control cells (Fig. 4A). Biochemical analysis showed a slight increase in ERK1/2 phosphorylation compared with control cells. In addition, a

Figure 4 Senescence programme is induced by oncogenic RET/PTC1 in primary human thyrocytes. Primary human thyrocytes transduced with retroviral vector carrying RET/PTC1 oncogene (Borrello et al. 2005) were analysed for the presence of OIS markers at passages 10–12; uninfected cells were used as control. (A) Analysis of cell morphology (upper panel; magnification 10×), SA-β-Gal activity (middle panel; magnification 10× for vector and 20× for RET/PTC1) and SAHFs formation (bottom panel; magnification 40×). Percentage of cells positive for SA-β-Gal activity and SAHFs are shown in each photograph. Cells enlarged to show DAPI-stained chromatin foci are indicated with arrows and shown in lower panel. (B) Western blot analysis of the indicated proteins in uninfected or infected cells. β-Actin was monitored as a loading control.
marked upregulation of p16\textsuperscript{INK4a}, p53 and HMGA2, and a slight increase of p21\textsuperscript{CIP1} was observed in RET/PTC1-infected cells (Fig. 4B).

To introduce TRK and RAS oncogenes into thyrocytes we employed the nucleofection technology, based on the evidence that BRAF\textsuperscript{V600E}-induced senescence in thyrocytes is detectable with this transfection method, as described earlier. Plasmid expression vectors carrying TRK-T3 or H-RAS\textsuperscript{G12V} oncogenes, and the empty vector as control, were transfected by nucleofection in primary human thyrocytes. Thyrocytes transfected with the TRK-T3 or H-RAS\textsuperscript{G12V} oncogenes underwent growth arrest and displayed flat, enlarged morphology and massive vacuolisation. Moreover, an increase of SA-\(\beta\)-Gal-positive cells was detected in TRK-T3 and H-RAS\textsuperscript{G12V} transfected cells (60 and 70\%, respectively), whereas in thyrocytes transfected with empty vector SA-\(\beta\)-Gal activity was observed in 10\% of cells. SAHFs were detected in 40 and 50\% of TRK-T3 and H-RAS\textsuperscript{G12V} transfected thyrocytes, respectively, but only in a small fraction (4\%) of control-transfected cells (Fig. 5A).

Biochemical analysis was performed at days 5 and 10 post-nucleofection (Fig. 5B). The expression of TRK-T3 and H-RAS\textsuperscript{G12V} was constant. An increase in ERK1/2 phosphorylation level, with respect to control cells, was induced by both oncoproteins at the two time points analysed. Similarly, an increase in p16\textsuperscript{INK4a} expression was observed. None of the oncogenes affected the levels of p21\textsuperscript{CIP1}. A slight increase of p53 was observed in H-RAS\textsuperscript{G12V} transfected cells at day 10.

Overall, our in vitro data suggests that all the PTC-associated oncogenes are capable of triggering senescence in primary human thyrocytes, and this is not related to the oncprotein expression level.

Analysis of OIS markers in thyroid tumour samples

To study the relevance of OIS in thyroid tumour pathogenesis we performed IHC analysis for the detection of OIS markers in a series of thyroid tumour samples characterised by increasing aggressiveness, thus representing epithelial thyroid tumour progression. The case collection included two PTMCs, seven PTCs (six NOS and one SV (Nikiforov et al. 2001)), two PDTC and one ATC. Clinical–pathological features and follow-up information for these cases are reported in Table 1. Notably, for PTMC #2 three intrathyroidal spreads and a nodal metastasis were available. Sample #9 (SV) and sample #10 (PDTC) presented areas with papillary and solid pattern of growth, thus providing a model of in situ tumour progression. The case collection was analysed by IHC for the expression of p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1}, well-known effectors of in vitro OIS, of IGFBP7, recently proposed as a factor required for OIS in melanocytes, and of the proliferation marker Ki-67. The results of IHC analysis are reported in Table 2; representative pictures are shown in Figs 6 and 7.

PTMC samples showed the highest expression of p16\textsuperscript{INK4a}, which was upregulated in tumour areas with respect to the adjacent non-tumoural thyroid tissue;
p16\textsuperscript{INK4a}-positive cells accounted for 50–60% of total. Immunolabelling for p16\textsuperscript{INK4a} was both cytoplasmic and nuclear. In PTMC #2 p16\textsuperscript{INK4a} was expressed at a similar level in all the different tumour areas analysed, including nodal metastasis. The PTCS NOS samples showed a variable extent of p16\textsuperscript{INK4a} expression: one case was scored negative (<10% of positive cells); four cases showed 11–40% of positive cells, whereas only one case displayed more than 40% positive cells. In general, the distribution of p16\textsuperscript{INK4a} was scattered; we observed groups of positive cells within the tumour mass. The PTC SV sample was scored positive in the areas with papillary growth, but negative in the solid ones. With respect to PTDC samples the papillary areas of case #10 were positive, whereas the solid areas as well as case #11 were negative. No expression of p16\textsuperscript{INK4a} was detected in the ATC sample.

### Table 2 Immunohistochemical analysis for p16\textsuperscript{INK4a}, p21\textsuperscript{CIP1}, IGFBP7 and Ki-67 in thyroid carcinomas

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>p16\textsuperscript{INK4a}</th>
<th>p21\textsuperscript{CIP1}</th>
<th>IGFBP7</th>
<th>Ki-67</th>
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<tbody>
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<td></td>
<td>+ + + +</td>
<td>+ + + + +</td>
<td>+</td>
<td>L</td>
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<tr>
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</tr>
<tr>
<td>2</td>
<td>PT, RL</td>
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<tr>
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<td>ITS, RL</td>
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<td>2</td>
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<td>+ + + + +</td>
<td>+</td>
<td>L/M</td>
</tr>
<tr>
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<td>Solid</td>
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<td>ND</td>
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<tr>
<td>PTC</td>
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<tr>
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<td>11</td>
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<tr>
<td>12</td>
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</table>

PTMC, papillary thyroid microcarcinoma; PTC, papillary thyroid carcinoma; ATC, anaplastic thyroid carcinoma; NOS, not otherwise specified; SV, solid variant; PT, primary tumour; ITS, intrathyroidal spread; LNM, lymph node metastasis; RL, right lobe; LL, left lobe; Is, isthmus. The percentage of cells with staining for p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1} was scored from 0 to 3: (−) <10% of positive cells; (1+) 11–40%; (2+) 41–70%; (3+) 71–100%. The intensity of staining for IGFBP7 was from: (−) negative; (+) weakly positive; (++) moderately positive; (+++) strongly positive. Ki-67 staining was scored as very low (vL, 1–5% positive cells), low (L, 6–10% positive cells), medium (M, 11–30% positive cells) and high (H, ≥31% positive cells). ND, not done (no material available).

With respect to p21\textsuperscript{CIP1}, no staining was observed in the non-tumoural tissue surrounding the tumour area. The highest expression was detected in PTMCs, showing 30–70% of positive cells, which persisted in the different lesions of PTMC #2. PTCs NOS were classified as negative, except for two cases that showed 11–40% of positive cells. The SV sample was scored positive in papillary areas and negative in solid ones. In PTDC, expression of p16\textsuperscript{INK4a} was detected only in the papillary areas of sample #10. No expression of p21\textsuperscript{CIP1} was observed in the ATC sample.

IGFBP7 showed the same trend of expression as p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1}. In both PTMC primary tumour samples, and in the intrathyroidal and nodal spreads of PTCM #2, IGFBP7 was upregulated with respect to the adjacent non-tumoural tissue. PTCS NOS showed variable expression levels: three cases were scored as moderately positive, one case as weakly positive, and two as negative. IGFBP7 expression was detected in the papillary, but not in the solid areas of the SV sample; a similar trend was observed for PDTC #10, whereas PDTC #11 showed weak expression, and no expression was detected in the ATC sample. The expression of p16\textsuperscript{INK4a}, p21\textsuperscript{CIP1} and IGFBP7 proteins was inversely correlated with the proliferative index. The expression of the Ki-67 proliferation marker was very low or low in PTMCs; it ranged from low to medium in PTC NOS and in the papillary areas of samples #9 and #10; it increased to medium/high and high in PDTC and ATC respectively. In Fig. 6A representative pictures of PTMC, PTC NOS, PDTC and ATC are shown; Fig. 6B reports the analysis of papillary and solid areas of PDTC sample #10. Interestingly, in PTC samples the expression of OIS markers was often associated with several structures suggestive of tumour regression (Fig. 6C) such as: i) areas with lymphocyte infiltration (left panel), which has been reported to play a protective role in thyroid cancer (Ugolini et al. 2007); ii) swelling oedematous papillae cores (middle panel) and iii) syncytial clusters of tumoural cells (right panel), a frequent finding in PTC (Fulciniti et al. 2001), resulting from papillae apex detachment.

The first conclusion that can be drawn from the IHC analysis is that the expression of OIS markers is upregulated at early stages and then lost during thyroid tumour progression. This issue is strengthened by the evidence that in samples #9 and #10, which recapitulate in situ tumour progression, the expression of the three proteins analysed was detected in areas featuring papillary growth but not in solid ones.

The highest expression of all OIS markers analysed observed in PTMCs would suggest that OIS may play a role in the generally indolent clinical course of this
PTC variant by keeping proliferation under control, even in the presence of intrathyroidal and nodal spreads, as suggested by the analysis of PTMC #2 (Table 2 and Fig. 7). To further explore this issue we analysed four additional PTMCs presenting with intraglandular and/or nodal spreads (see Table 1); due to material shortage the analysis was limited to Ki-67 and p16INK4a. In Table 3, the primary tumours showed relatively intense p16INK4a expression and very low Ki-67 immunolabelling. The same pattern of expression was observed in the intraglandular and/or lymph node tumour foci. Interestingly, in none of the PTMC patients was recurrence documented in the follow-up period, which ranged from 72 to 102 months (Table 1). Overall, our data demonstrate the presence of OIS in PTMC, and suggests that this mechanism maintains the indolent behaviour of this tumour type, despite multifocality within thyroid gland and loco-regional spread.

**Discussion**

We provided evidence that OIS may represent a barrier to the progression of thyroid tumours. *In vitro* studies, using different gene transfer methods, demonstrated that all the oncogenes activated in thyroid tumours, namely *BRAFV600E*, *RET/PTC1*, *TRK-T3* and *H-RASG12V*, are capable of inducing cellular senescence in primary human thyrocytes, as judged by the presence of growth arrest, changes in cell morphology, accumulation of SA-β-Gal and chromatin modifications. Our conclusion is corroborated by the evidence that OIS is detectable with different oncogenes transfected with different methods and, thus, expressed at different levels. Differences in efficiency and onset of OIS observed with the different oncogenes can be ascribed to experimental conditions and oncoprotein expression level, rather than to intrinsic oncogenic features.

Shedding light on the molecular mechanisms responsible for the OIS onset, the predominant view is that aberrant levels of ERK1/2 activate a senescence programme through the critical involvement of the p16INK4a/pRB and p53/p21CIP1 networks (Lin et al. 1998). In our cellular setting, PTC-associated oncogenes trigger a marked activation of ERK1/2, accompanied by a persistent induction of p16INK4a, whereas we failed to observe any significant upregulation of p53 or p21CIP1, with the exception of...
RET/PTC1-expressing thyrocytes in which an increase of p53 was detected. We observed a reduction in IGFBP7, whose role in OIS is debated (Scurr et al. 2010).

The relative contribution of the p16 INK4a/pRB and the p53/p21 CIP1 pathways in mediating a senescence programme remains poorly understood. It has been shown that p16 INK4a plays a more major role than p21 CIP1 in the induction of senescence in many contexts in vitro. In some cells, p16 INK4a is solely responsible for OIS onset (Ben-Porath & Weinberg 2005), whereas in other cell types it has been shown that p16 INK4a alone is not sufficient to execute the OIS programme (Michaloglou et al. 2005, Haferkamp et al. 2009). In our experimental setting the inactivation of p16 INK4a with shRNA did not affect BRAFV600-induced senescence in thyrocytes, except for a reduction of SAHF-bearing cells. This raises the possibility that p16 INK4a may cooperate with other factors in triggering senescence.

The upregulation of p16 INK4a observed in our analysis is in agreement with previous studies, showing that the proliferation arrest induced in thyrocytes by H-Ras G12V was associated with upregulation of p16 INK4a (Jones et al. 2000). In that setting, however, silencing p16 INK4a rescued H-Ras G12V expressing thyrocytes from senescence (Bond et al. 2004), suggesting that the OIS programme might not be universal in different genetic contexts.

Although initially considered as an in vitro phenomenon, OIS is now viewed as an authentic cancer barrier. Several groups have reported its occurrence in different in vivo lesions including human melanocytic nevi, human dermal neurofibromas, human and murine prostatic and thyroid adenomas, and murine lymphomas and melanoma (Braig et al. 2005, Chen et al. 2005, Collado et al. 2005, Courtois-Cox et al. 2006, Kortlever et al. 2006, Dankort et al. 2007, Ha et al. 2007). To extend our observations to an in vivo context we analysed a panel of thyroid tumour samples with increasing aggressiveness: PTMC, characterised by a very indolent course; PTC NOS, a differentiated carcinoma generally associated with a good prognosis; PDTC, associated with a poor prognosis; ATC, an undifferentiated, very aggressive carcinoma with fatal outcome, mostly arising from a time-dependent dedifferentiation of differentiated carcinoma, in particular PTC. We analysed the expression of p16 INK4a and p21 CIP1, two universally recognised OIS markers, and IGFBP7, a protein with an oncosuppressor role in thyroid tumours (Vizioli et al. 2010), although its role in this process is controversial (Scurr et al. 2010). Collectively, the three proteins were upregulated in
Table 3 Immunohistochemical analysis for p16\textsuperscript{INK4a} and Ki-67 in a small series of PTMCs

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sample</th>
<th>p16\textsuperscript{INK4a}</th>
<th>Ki-67</th>
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<tbody>
<tr>
<td>13</td>
<td>PT, Is</td>
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<td>vL/L</td>
</tr>
<tr>
<td>13</td>
<td>ITS, RL</td>
<td>+ +</td>
<td>vL</td>
</tr>
<tr>
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<td>ITS, LL</td>
<td>+</td>
<td>vL</td>
</tr>
<tr>
<td>14</td>
<td>PT, LL</td>
<td>+ +</td>
<td>vL</td>
</tr>
<tr>
<td>14</td>
<td>ITS, Is</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>LNM</td>
<td>+ +</td>
<td>vL</td>
</tr>
<tr>
<td>15</td>
<td>PT, RL</td>
<td>+ +</td>
<td>vL</td>
</tr>
<tr>
<td>15</td>
<td>ITS, RL</td>
<td>ND</td>
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</tr>
<tr>
<td>16</td>
<td>PT, RL</td>
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<td>vL</td>
</tr>
<tr>
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</tr>
<tr>
<td>16</td>
<td>ITS, LL</td>
<td>+</td>
<td>vL</td>
</tr>
</tbody>
</table>

PT, primary tumour; ITS, intrathyroidal spread; LNM, lymph node metastasis; RL, right lobe; LL, left lobe; Is, isthmus. The percentage of cells with staining for p16\textsuperscript{INK4a} was scored from 0 to 3: (−) <10% of positive cells; (1+) 11–40%; (2+) 41–70%; (3+) 71–100%. Ki-67 staining was scored as very low (vL, 1–5% positive cells), low (L, 6–10% positive cells), medium (M, 11–30% positive cells) and high (H, ≥31% positive cells). ND, not done (no material available).

PTMCs; the PTCs showed a variable pattern, including loss of expression; finally, the ATC sample was scored negative for all the proteins. The upregulation in PTMCs and the progressive loss in PTCs and ATC support the notion that OIS may counteract oncogenic activity in thyroid tumours, and its escape allows thyroid tumour progression.

Regarding IGFBP7, we have previously shown that its mRNA expression is frequently downregulated in PTC, and this is associated with the TC variant (Vizioli et al. 2010). Our IHC results are in agreement with the gene expression data, as we observed a progressive loss of IGFBP7 protein during thyroid tumour progression. The trend of IGFBP7 expression was similar to that of p16\textsuperscript{INK4a} and p21\textsuperscript{CIP1}, suggesting that it may participate in counteracting the aberrant proliferative signals of oncogenes. However, in senescent BRAF\textsuperscript{V600E} thyrocytes we detected a reduction of IGFBP7, at both the protein and mRNA level. This discrepancy may be explained by taking into account that the induction of IGFBP7 \textit{in vivo} might result from the complex crosstalk between tumour and stromal cells (Kuillman & Peep 2009). Nevertheless, more studies are required to assess if IGFBP7 plays a role in thyrocyte OIS.

Further support to the suggestion that OIS counteracts oncogenic activation in PTC is provided by the evidence that the expression of OIS markers may be associated with several characteristics of tumour regression, such as lymphocytic infiltration, oedematous papillae cores and syncytial clusters. The presence of these features is in keeping with the notion that OIS triggers the activation of the inflammatory network and evokes an innate immune response that ultimately counteracts tumour progression (Ren et al. 2009).

Analysis of a small series of PTMC showed the consistent upregulation of p16\textsuperscript{INK4a} in association with a very low proliferative index. Of note, p16\textsuperscript{INK4a} expression and Ki-67 immunolabelling remained unchanged in synchronous intrathyroidal and nodal spreads. In one PTMC sample, the analysis of p21\textsuperscript{CIP1} and IGFBP7 was possible, showing the same expression level in the primary tumour, and intrathyroidal and nodal foci. Notably, none of the PTMC patients of our study experienced recurrence during the follow-up period (72–102 months).

Expression of p16\textsuperscript{INK4a} in thyroid malignancies has been previously investigated by several groups. Overexpression of p16\textsuperscript{INK4a} in adenomas, papillary and follicular tumours, and the loss of expression in ATC was observed in previous works (Ferru et al. 2006, Ball et al. 2007), suggesting that p16\textsuperscript{INK4a} expression is induced in early-stage tumours and then progressively lost during tumour progression. Similarly, the overexpression of p21\textsuperscript{CIP1} in PTMC and progressive loss of expression with advancing tumour grade in PTC has been reported (Brzezinski et al. 2005). These previous results confirm our data and suggest that the indolent course of PTMC, as well as the characteristically slow growth of classical PTC, may be explained by the persistence of a severe limit to oncogene-induced proliferation exerted by the OIS mechanism. In this light PTMC represents, among the variants examined, the most representative model for the tumour suppressor mechanism exerted by OIS. Indeed, all the foci examined across the thyroid and lymph nodes share the same ‘frozen phenotype’ in terms of p16\textsuperscript{INK4a}, p21\textsuperscript{CIP1} and IGFBP7.

The persistence of an OIS signal in intrathyroidal and nodal spreads might explain the favourable prognosis of PTMC even in the presence of multifocality and lymph node metastasis, two features associated with lateral node recurrence, but not with an increased risk of mortality (Hay et al. 2008).

Moreover, the constant expression of all the OIS markers analysed and Ki-67 labelling across the different lesions of the same patient suggests that cells have acquired the capability to invade without changes in their proliferation capability. This is in keeping with reports claiming invasion and proliferation as two distinct mechanisms (Gao et al. 2005), governed by different genes and pathways.

We have identified OIS as a mechanism involved in the pathogenesis of thyroid carcinoma by restraining the evolution of PTMC to PTC, as well as the transition...
of well differentiated and less aggressive PTC to undifferentiated and more aggressive variants. This raises the idea that restoration of this process in tumour cells may represent a therapeutic approach, especially for those tumours which do not respond to standard therapy. Moreover, a possible therapeutic strategy based on OIS restoration is independent from the oncogenic alteration carried by the tumour, as we have shown that all PTC-associated oncogenes are capable of triggering the senescence programme. The feasibility of enforced cellular senescence as a therapeutic approach for thyroid as well as other tumour types remains to be investigated, and it will require a deeper understanding of the mechanisms involved in the process, as well as suitable in vitro and in vivo models.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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