Impairment of the p27kip1 function enhances thyroid carcinogenesis in TRK-T1 transgenic mice

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Abstract

Impairment of the p27kip1 function, caused by a drastic reduction of its expression or cytoplasmic mislocalization, has been frequently observed in thyroid carcinomas. To understand the role of p27kip1 impairment in thyroid carcinogenesis, we investigated the consequences of the loss of p27kip1 expression in the context of a mouse modeling of papillary thyroid cancer, expressing the TRK-T1 oncogene under the transcriptional control of thyroglobulin promoter. We found that double mutant mice homozygous for a p27kip1 null allele (TRK-T1/p27kip1/p27kip1) display a higher incidence of papillary thyroid carcinomas, with a shorter latency period and increased proliferation index, compared with p27kip1 wild-type compounds (TRK-T1/p27kip1/p27kip1). Consistently, double mutant mice heterozygous for a p27kip1 null allele (TRK-T1/p27kip1/p27kip1) show an incidence of thyroid carcinomas that is intermediate between TRK-T1/p27kip1/p27kip1 and TRK-T1/p27kip1/p27kip1 mice. Therefore, our findings suggest a dose-dependent role of p27kip1 function in papillary thyroid cancer development.

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Introduction

Thyroid tumors include a wide spectrum of lesions with different phenotypic characteristics and biological behavior: benign adenomas, differentiated carcinomas, and anaplastic carcinomas (Hedinger et al. 1989). Several genetic lesions have been already identified in human thyroid carcinomas. In fact, RET/PTC onco-genes have been found in about 20% of human papillary thyroid carcinomas (PTCs). They are chimeric genes generated by the fusion of the catalytic domain of the RET receptor tyrosine kinase with the N-terminal region encoded by heterologous genes. In the most prevalent variants, RET/PTC1 (Greco et al. 1990) and RET/PTC3 (Santoro et al. 1994), the fusion occurs between RET and the H4 (D10S170) or RFG (Ele1/ARA70/Ncoa4) genes respectively. TRK-T onco-genes have been detected in about 5% of PTCs. They are generated by structural rearrangements of the NTRK1 (TRKA) gene, coding for the high affinity nerve growth factor (NGF) receptor. The TRK-T1 and TRK-T2 onco-genes are both generated by NTRK1 fusion with the TPR gene. NTRK1 and TPR are both located on human chromosome 1 (Greco et al. 1997). Recently, it has been demonstrated that specific point mutations in the activation segment of the BRAF kinase are present in about 40% of adult PTCs (Kimura et al. 2003). Furthermore, a novel onco-gene, designated AKAP9-BRAF, deriving from the in-frame fusion of the first eight exons of the A-kinase anchor protein 9
(AKAP9) gene with the carboxyl-terminal encoding-region (exons 9–18) of BRAF, has been isolated from PTCs developed in irradiated patients after a short latency period (Ciampi et al. 2005). As far as ras genes are concerned, mutations of codon 61 of N-RAS (N2) were significantly more frequent in follicular tumors (19%) than in papillary cancers (5%). H-RAS mutations in codons 12/13 (H1) were found in 2–3% of all types of thyroid tumors, but H-RAS mutations in codon 61 (H2) were observed in only 1.4% of tumors (Vasko et al. 2003). K-RAS mutations in exon 1 were found more often in papillary carcinomas (De Micco 2003). The genetic lesions described above are mutually exclusive, since no tumors have been found bearing more than one of these lesions. PAX8-PPAR-γ rearrangements are frequent in follicular thyroid carcinomas (Kroll et al. 2000, Dwight et al. 2003), whereas impaired function of the p53 tumor suppressor gene is a feature of anaplastic thyroid carcinomas (Donghi et al. 1993). It is interesting to observe that all these oncogenes identified in thyroid cancer activate a signaling pathway that results in an increased MAPK activity, according to which one specific signaling pathway that leads to MAPK activation plays a major role in the generation of PTC. However, other genetic lesions have also been reported in PTCs: decreased levels of PTEN and PTPRJ, a dual-specific phosphatase and a receptor type tyrosine phosphatase respectively (Bruni et al. 2000, Trapasso et al. 2000), and alterations of the p27kip protein function (Baldassarre et al. 1999) represent frequent features of thyroid malignancies.

p27kip1 has classically been regarded as a cell-cycle inhibitor based on its potent inhibitory activity of cyclin E/cdk2 and the observation that its forced expression results in G1 arrest (Toyoshima & Hunter 1994). Low levels of the p27kip1 protein have been frequently found in human carcinomas, and these low levels directly correlate with both a high histological tumor grade and a poor outcome (Lloyd et al. 1999). In several cases, the impairment of the p27 function is due to its cytoplasmic mislocalization induced by AKT activation (Viglietto et al. 2002, Motti et al. 2005, Chu et al. 2008). Also in thyroid malignant neoplasias, the impairment of the p27 function is very frequent: a reduction in p27kip1 protein levels has been previously described in 10 out of 28 papillary carcinomas, 3 out of 9 follicular carcinomas, and 6 out of 8 anaplastic carcinomas. Moreover, 80% of p27kip1-expressing tumors shows an uncommon cytoplasmic localization of p27kip1 protein, associated with a high cdk2 activity (Baldassarre et al. 1999). Several components account for the reduced p27 protein levels in thyroid malignant neoplasias: both PTEN and PTPRJ, whose expression is drastically reduced in thyroid malignant neoplasias (Bruni et al. 2000, Trapasso et al. 2000), increase the stability of the p27 protein. Recent data show increased miR221 and 222 levels in PTCs that correlate with low levels of p27 (Visone et al. 2007). However, a causal link between p27 impairment and thyroid carcinogenesis has not been established yet.

The aim of the present work was to validate the role of the loss of p27 function in thyroid carcinogenesis crossing transgenic mice carrying the TRK-T1 transgene under the control of the bovine thyroglobulin (TG) promoter (Russell et al. 2000) with mice carrying targeted germine deletions of one or two p27kip1 alleles (Fero et al. 1996). Despite the limited involvement of the NTRK gene in human PTCs in comparison with RET/PTC, the TRK-T1 mouse model develops follicular hyperplasia and papillary carcinoma similarly to RET/PTCs mice (Jiang et al. 1996, Santoro et al. 1996, Powell et al. 1998, Russell et al. 2000), thus representing a valid system in the study of thyroid carcinogenesis mechanisms. p27 null mice show a syndrome of multigain hyperplasia with features of gigantism and develop pituitary adenoma with high incidence, lung, gonadal, and intestinal tumors at an increased frequency compared with wild-type mice (Fero et al. 1996), but no thyroid tumors or anomalies. Here, we report that loss-of-function of p27kip1 in mice with targeted overexpression of TRK-T1 in thyroid cells increases the penetrance of thyroid cancer and shortens the latency period of tumor incidence.

Materials and methods

Mouse strains, handling, and genotyping

TRK-T1 transgenic mice in a B6C3F1 genetic background (Russell et al. 2000) were crossed with p27kip1 null (p27−/−) mice from a mixed C57BL/6J and 129 genetic background (from Dr M L Fero; Fero et al. 1996) to obtain p27+/+, p27+/−, and p27−/− mice containing the TRK-T1 transgene (TRK-T1/p27+/+, TRK-T1/p27+/−, and TRK-T1/p27−/− respectively). The TRK-T1 and p27kip1 genotypes were analyzed by the PCR with genomic DNA isolated from ~1 cm section of mouse tail. For p27kip1 genotyping, the following PCR primers were used: p27-K3 (common) TGGAACCTGTGCCATCTCATT; p27 F-N (generates mutant fragment = 800 bp), CCTTCTATGG-CCTTCTTGACG; and p27-K5 (generates wild-type fragment = 900 bp), GAGCAGACGCCCAAGAAGC. The primer sequences for TRK-T1 were forward,
protein extracts were separated by SDS-PAGE and transferred to nitrocellulose membranes (BioRad). Membranes were blocked with 5% nonfat milk in TTBS (Tris-buffered saline containing Tween-20) and incubated with antibodies diluted in the same solution. Bound antibodies were detected by the appropriate HRP-conjugated secondary antibodies followed by enhanced chemiluminescence (Amersham).

Statistical analysis
Kaplan–Meyer survival curves were used to analyze the percentage of tumor-free mice in all groups examined during the 20 months of monitoring. Differences were analyzed by Logrank test. For the comparison of cases and controls within and across the groups, normal, hyperplastic or tumoral frequencies were calculated for all participants and compared using $\chi^2$ analysis. Ki67 data are expressed as mean $\pm$ S.E.M. Differences were analyzed by Student’s $t$-test. $P$ values $\leq 0.05$ were considered significant for all the above statistical assays.

Results
The onset of PTCs is accelerated in p27-null mice
Mice carrying the TRK-T1 transgene under the control of the bovine TG promoter (Russell et al. 2000) were mated with mice carrying targeted germline deletions of one or two p27$^{kip1}$ alleles (Fero et al. 1996). From the above intercross, mice of all the three genotypic combinations (TRK-T1/p27$^{+/+}$, TRK-T1/p27$^{+-/-}$, and TRK-T1/p27$^{-/-}$) were obtained, and cohorts of 40 TRK-T1/p27$^{+/+}$, 52 TRK-T1/p27$^{+-/-}$, and 40 TRK-T1/p27$^{-/-}$ plus 20 wild-type and 20 p27$^{-/-}$ mice were included in our study and monitored daily for the appearance of a thyroid phenotype. Each mouse was killed at the gross observation of a neck mass and within 20 months of age, all the remaining mice were killed. Histopathological analyses of each thyroid lobe were performed on thyroids of all mice. On the strength of criteria previously described (Russell et al. 2000), we classified all the specimens in three categories: normal, hyperplasia, and papillary carcinoma. As shown in Fig. 1, differently from normal thyroid characterized by regular monolayered follicles containing colloid (panel A), hyperplasia was characterized by increased follicular cellularity together with the presence of irregular or colloid-deficient follicles (panel B), whereas PTC was diagnosed on the presence of proliferation of follicular epithelial cells containing scant cytoplasm and papillae containing fibrovascular stalks (panel C). As shown in Fig. 2A, we found that TRK-T1/p27$^{-/-}$ develop PTCs more precociously.

Histological and immunohistochemical procedures
Cohorts of 40 TRK-T1/p27$^{+/+}$, 52 TRK-T1/p27$^{+-/-}$, and 40 TRK-T1/p27$^{-/-}$ live mice, equally distributed between males and females, were examined for tumor formation by observation and palpation every week beginning 1 month after birth. They were killed at the gross appearance of a neck mass or at 20 months of age and thyroid was removed for pathological analysis. In parallel, cohorts of 18 mice for each of the above genotypes, equally distributed between males and females, were killed in a time window of 12–14 months of age and their thyroids histologically examined. Tissues were fixed by immersion for 24 h in Bouin’s solution and embedded in paraffin using standard procedures. Sections (5 µm) were stained with hematoxylin and eosin using standard histological techniques. To determine the proliferative status, tumor sections were incubated with monoclonal antibody to Ki-67 (Dako, Milano, Italy) at a 1:15 dilution following microwave antigen retrieval with citrate buffer on an autostainer (Dako) using the LSAB + kit (Dako). The percentage of apoptotic cells in the same tumor sections was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) using the TdT-FragEL DNA fragmentation Detection Kit (Calbiochem, Beeston, Nottinghamshire, UK). Micrographs were taken on Kodak Ektachrome film with a photo Zeiss system.

Protein extraction and western blot analysis
Frozen pieces from normal and tumoral thyroid samples were resuspended in NIH lysis buffer (1% Nonidet P-40; 1 mM EDTA; 50 mM Tris–HCl (pH 8.0); 150 mM NaCl; 2 mM phenylmethysulfonyl fluoride; 50 mM NaF; 10 mM Na$_3$VO$_4$; 20 mM NaPP; 1.5 mM aprotinin). Tissue lysates were clarified by centrifugation at 10 000 $g$ for 20 min at 4 °C. The supernatant was then used for immunoblotting. Western blotting was performed by standard procedures using the following primary antibodies: anti-p27 (sc-528; Santa Cruz, Heidelberg, Germany), anti-trk (sc-11; Santa Cruz), and anti-$\gamma$-tubulin (sc-8035; Santa Cruz). Briefly, the
as compared with compound TRK-T1/p27+/+ and TRK-T1/p27−/− mice. In particular, the comparison of tumor incidence curves (here representing the PTC-free mice) by Logrank test showed highly significant differences between TRK-T1/p27−/− and TRK-T1/p27+/+ mice (P < 0.0001). Similarly, significant differences were found between TRK-T1/p27+/− and TRK-T1/p27+/+ mice (P = 0.0328), as well as between TRK-T1/p27−/− and TRK-T1/p27+/− mice (P = 0.0348). No wild-type or p27−/− mice, lacking the TRK-T1 transgene expression, develop PTCs by 20 months of age (Fig. 2A, dotted line).

Loss of p27 confers an increased proliferation rate to PTCs

Histological analyses of the PTCs arising in TRK-T1/p27+/+, TRK-T1/p27+/−, and TRK-T1/p27−/− mice did not show any significant differences and no
abnormalities or tumor metastases in local cervical lymph nodes, peripheral lymph nodes or lungs were observed in any group (data not shown). Then, we analyzed in a comparative manner the proliferation index in PTCs developed by TRK-T1 mice homozygous, heterozygous or wild type for the p27 gene. The proliferation marker Ki67 was increased in PTC cells from both TRK-T1/p27\(^{+/c}\) (23.5\% \pm 2.1) and TRK-T1\(^{-/-}\) (25\% \pm 2) mice compared with p27 wild-type compounds (9\% \pm 1.7; Fig. 3 and data not shown). Regardless of p27 dosage, Trk-induced PTCs contained a similar percentage of apoptotic cells (as determined by TUNEL analysis) as did normal thyroid tissues (data not shown). These findings indicate that loss of p27\(^{kip1}\) enhances tumor growth by increasing the percentage of cycling cells (rather than the percentage of surviving cells) in the tumors.

Finally, to exclude the possibility that the loss of p27\(^{kip1}\) may act on the transgene expression, leading to an increased mitogenic stimulus, thus assessing that the increased malignant phenotype is caused by the impairment of the p27 function, we have analyzed the transgene expression in PTCs from two mice for each genotypic combination. As shown in Fig. 4, no significant differences have been observed in TRK protein expression between p27 null and p27 wild-type mice. The expression of the p27 protein was also analyzed as control.

Discussion

Decreased expression and altered subcellular localization of p27 have been observed in thyroid cancers (Motti et al. 2005), but the functional relevance of this observation in vivo is not addressed yet. For this reason, we used a mouse experimental model of thyroid carcinogenesis to investigate in vivo the role of p27 inactivation in such neoplasm. In particular, we used transgenic mice for the TRK-T1 gene, a well-described murine model of thyroid carcinogenesis (Russell et al. 2000), to investigate the relationship between the overexpression of the TRK-T1 transgene and p27\(^{kip1}\) deletion in inducing thyroid carcinomas. Here, we report that the loss of p27\(^{kip1}\)
function increases the malignant phenotype of mice expressing the TRK-T1 oncogene under the transcriptional control of TG promoter. Indeed, the TRK-T1/p27\(^{-/-}\) mice show an increased number of carcinomas appearing with a shorter latency period compared with TRK-T1/p27\(^{+/+}\) mice. The analysis of PTCs arising in TRK-T1/p27\(^{-/-}\) mice shows an increased Ki67 positivity that indicates an increased proliferation rate in comparison with the PTCs arising in compound p27 wild type. Conversely, TRK-T1/p27\(^{+/+}\) mice show a phenotype that can be considered intermediate between TRK-T1/p27\(^{-/-}\) and TRK-T1/p27\(^{+/+}\) mice: their incidence of thyroid carcinomas was higher than TRK-T1/p27\(^{+/+}\) mice, but lower than TRK-T1/p27\(^{-/-}\) mice. Equally, TRK-T1/p27\(^{+/+}\) mice have an incidence of PTCs that was earlier than TRK-T1/p27\(^{+/+}\) mice, but significantly later than TRK-T1/p27\(^{-/-}\) mice. Finally, PTCs developing in these mice show a proliferative index statistically increased compared with that shown by PTCs arising in TRK-T1/p27\(^{+/+}\) mice, but lower than TRK-T1/p27\(^{-/-}\) mice.

Interestingly, our results appear in contrast with those previously published regarding the p27-null mice carrying the ErbB2/Neu oncogene (MMTV-neu/p27\(^{-/-}\); Muraoka et al. 2002). The mammary glands of these mice showed decreased proliferation, as well as markedly prolonged tumor latency, compared with MMTV-neu/p27\(^{+/+}\) glands. Conversely, the TRK-T1/p27-null mice showed a great similarity with the MMTV-neu/p27\(^{-/-}\) mice, whose mammary glands exhibited alveolar hyperplasia, enhanced proliferation, decreased apoptosis, and accelerated tumor formation compared with MMTV-neu/p27\(^{+/+}\) mammary glands (Muraoka et al. 2002).

It is likely that in MMTV-neu/p27\(^{-/-}\) mice, the complete absence of the p27 protein leads to an impairment of cyclin D1/Cdk4 function, that makes these mice more resistant to transformation. Results very similar to those described for the MMTV-neu/p27\(^{-/-}\) and MMTV-neu/p27\(^{+/+}\) have also been described in a mouse model of prostate carcinogenesis (Gao et al. 2004): in p27 minus mice there is an inhibition of tumor progression, whereas the heterozygous compounds display enhanced prostate carcinogenesis. However, we must consider that p27 plays both a permissive and an inhibitory role in normal thyroid cell proliferation (Paternot et al. 2006), suggesting that also in carcinomas it can behave in both ways depending on the cellular context.

**Figure 3** Ki67 staining of PTCs arising in TRK-T1/p27\(^{+/+}\) and TRK-T1/p27\(^{-/-}\) mutants. (A and B) Representative Ki67 immunostaining of PTCs occurring in TRK-T1/p27\(^{+/+}\) and TRK-T1/p27\(^{-/-}\) mice. A higher positivity is evident in the latter specimen (B) as better described in the text. Bar scale = 100 μm.

**Figure 4** Western blot analyses on PTCs arising in TRK-T1 mice with different p27 genotypes. The proteins detected are indicated on the side. The γ-tubulin protein expression was analyzed as a loading control. Two normal thyroids from wild-type animals were also included as control.
Therefore, the results shown here demonstrate that loss of p27 has a role on thyroid tumor development and that p27 behaves as a haploinsufficient tumor suppressor gene since even the loss of only one allele of p27 is sufficient to confer a proliferative advantage to PTC growth.

Declaration of interest

We state that there does not exist any potential conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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