Impairment of the p27^{kip1} function enhances thyroid carcinogenesis in *TRK-T1* transgenic mice

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Abstract

Impairment of the p27^{kip1} function, caused by a drastic reduction of its expression or cytoplasmic mislocalization, has been frequently observed in thyroid carcinomas. To understand the role of p27^{kip1} impairment in thyroid carcinogenesis, we investigated the consequences of the loss of p27^{kip1} 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 p27^{kip1} null allele (*TRK-T1/p27*^{-/-}) display a higher incidence of papillary thyroid carcinomas, with a shorter latency period and increased proliferation index, compared with p27^{kip1} wild-type compounds (*TRK-T1/p27*^{+/+}). Consistently, double mutant mice heterozygous for a p27^{kip1} null allele (*TRK-T1/p27*^{+/-}) show an incidence of thyroid carcinomas that is intermediate between *TRK-T1/p27*^{-/-} and *TRK-T1/p27*^{+/+} mice. Therefore, our findings suggest a dose-dependent role of p27^{kip1} 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 oncogenes 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 (Grieco *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* oncogenes 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* oncogenes 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 oncogene, 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 *p27^{kip1}* protein function (Baldassarre et al. 1999) represent frequent features of thyroid malignancies.

p27^{kip1} 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 *p27^{kip1}* 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 *p27^{kip1}* 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 *p27^{kip1}*-expressing tumors shows an uncommon cytoplasmic localization of *p27^{kip1}* 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 germline deletions of one or two *p27^{kip1}* 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 (Jhiang 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 multiorgan 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 *p27^{kip1}* 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 *p27^{kip1}*-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 *p27^{kip1}* genotypes were analyzed by the PCR with genomic DNA isolated from ~1 cm section of mouse tail. For *p27^{kip1}* genotyping, the following PCR primers were used: *p27*-K3 (common) TGGAACCTGTGCCATCTCTAT; *p27* F-N (generates mutant fragment=800 bp), CCTTCTATGG-CCTTCTTGACG; and *p27*-K5 (generates wild-type fragment=900 bp), GAGCAGACGCCAAGAAGC. The primer sequences for TRK-T1 were forward,

CACATCATCGAGAACCCACAA and reverse, GCTCATGCCAAAATCACCAAT, which generate a 550 bp product.

All mice were maintained under specific pathogen-free conditions in our Laboratory Animal Facility (Istituto dei Tumori di Napoli, Naples, Italy) and all studies were conducted in accordance with Italian regulations for experiments on animals.

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 phenylmethylsulfonyl fluoride; 50 mM NaF; 10 mM Na₃V₄; 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-γ-tubulin (sc-8035; Santa Cruz). Briefly, the

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 χ^2 analysis. Ki67 data are expressed as mean $\pm$ S.E.M. Differences were analyzed by Student's *t*-test. *P* values ≤ 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

Figure 1 Histopathological features of normal, hyperplastic, and carcinomatous mouse thyroids. (A) Representative example of a normal thyroid as it appeared in a wild-type mouse. Uniform colloid-filled follicles, composed of a one cell thick layer of cuboidal epithelium are present. (B) Representative example of a hyperplastic thyroid lesion as it appeared in a *TRK-T1/p27^{+/-}* mouse. Increased numbers of thyroid follicular epithelial cells surrounding large colloid-deficient follicles are present. (C) Representative example of PTC developed in a *TRK-T1/p27^{-/-}* mouse. Follicular epithelial cells cluster together forming continuous sheets or papillae that surround a fibrovascular stalk. The arrow indicates a fibrovascular stalk in a papillary structure. Bar scale = 100 μ m.

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).

p27 deficiency resulted in increased malignant thyroid transformation

On the basis of the histopathological results obtained from the above criteria on the thyroids of the mice killed in a time window of 12–14 months of age (18 mice for each genotype, equally distributed between males and females), we found significant pathological differences in both *TRK-T1/p27^{+/-}* and *TRK-T1/p27^{-/-}* mice compared with *TRK-T1/p27^{+/+}*. In particular, as summarized in Fig. 2B and analytically described in Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>, the total number of PTCs developed by *TRK-T1/p27^{-/-}* mice was significantly higher than those observed in both compounds heterozygous for the *p27* null mutation ($P < 0.005$) and wild type ($P < 0.0005$). Interestingly, the carcinoma incidence in *TRK-T1/p27^{+/-}* mice was statistically higher than in *TRK-T1/p27^{+/+}* ($P < 0.01$), but intermediate between compound *p27^{-/-}* and *p27^{+/+}* mice, indicating a dose-dependency of *p27* in TRK-induced tumor development (Fig. 2B). In addition, the data in Fig. 2B also show that *TRK-T1/p27^{+/-}* and *TRK-T1/p27^{-/-}* mice do not significantly differ from each other for the incidence of a normal phenotype, being in both of them significantly lower than in *TRK-T1/p27^{+/+}* mice ($P < 0.0005$). Consequently, differently from *TRK-T1/p27^{-/-}* mice ($P < 0.005$), compound mice heterozygous for the *p27*-null mutation show a high incidence of hyperplasias that likely represent a pre-malignant stage toward the development of PTCs, as suggested by the delayed onset of PTCs in *TRK-T1/p27^{+/-}* compared with *TRK-T1/p27^{-/-}* mice (Fig. 2A). No sex-related differences were observed (Supplementary Table 1). As a control, we also analyzed similar cohorts of wild-type and *p27^{-/-}* mice finding no thyroid transformation in any of them (Fig. 2B and Supplementary Table 1).

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

Figure 2 Deficiency of p27 alters cancer incidence of *TRK-T1* mice. (A) Kaplan–Meyer tumor incidence analysis of *TRK-T1* mice with different *p27* genotypes. Cohorts of at least 40 mice were monitored and harvested when they exhibited symptoms of disease. As assessed by Logrank test, the curves were significantly different ($P=0.0009$). Dotted line represents either wild-type or *p27*^{-/-} mice. (B) The incidence of mice bearing normal, hyperplastic or cancerous thyroid was reported for each genotype, including negative controls, during a time-window of 12–14 months of age. Differences between two frequencies were statistically compared using χ^2 analysis. * $P<0.005$; ** $P<0.0005$ versus *TRK-T1/p27*^{+/+} controls.

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*^{+/-} ($23.5\% \pm 2.1$) 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*^{kip1} 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}

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.

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 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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