MicroRNAs (miR)-221 and miR-222, both overexpressed in human thyroid papillary carcinomas, regulate p27^{Kip1} protein levels and cell cycle

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Abstract

We have recently reported that MicroRNAs (miR)-221 and miR-222 were up-regulated in human thyroid papillary carcinomas in comparison with the normal thyroid tissue. Bioinformatic analysis proposed the p27^{Kip1} protein, a key regulator of cell cycle, as a candidate target for the miR-221/222 cluster. Here, we report that the enforced expression of miR-221 and miR-222 was able to reduce p27^{Kip1} protein levels in thyroid carcinoma and HeLa cells in the absence of significant changes in specific p27^{Kip1} mRNA levels. This effect is direct as miR-221 and miR-222 negatively regulate the expression of the 3'-untranslated region-based reporter construct from the p27^{Kip1} gene, and is dependent on two target sites in this region. Consistent with these results, an enforced expression of the miR-221 and miR-222 induced the thyroid papillary carcinoma cell line (TPC-1) to progress to the S phase of the cell cycle. It is likely that the negative regulation of p27^{Kip1} by miR-221 and miR-222 might also have a role *in vivo* since we report an inverse correlation between miR-221 and miR-222 up-regulation and down-regulation of the p27^{Kip1} protein levels in human thyroid papillary carcinomas. Therefore, the data reported here demonstrate that miR-221 and miR-222 are endogenous regulators of p27^{Kip1} protein expression, and thereby, the cell cycle.

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Introduction

MicroRNAs (miRs) have emerged as an important class of short endogenous RNAs that act as post-transcriptional regulators of gene expression by base-pairing with their target mRNAs. A majority of identified miRs are highly evolutionarily conserved among many distantly related species suggesting that miRs play a very important role in essential biological processes, including developmental timing, stem cell differentiation, signaling transduction, cell growth, and cancer. Presently, miRs have been considered one of the most important regulatory

molecules, which regulate gene expression at the post-transcriptional levels by targeting mRNAs for direct cleavage or by repressing mRNA translation (Ambros 2004, Bartel 2004, Lewis *et al.* 2005).

By the analysis of the genome-wide miR expression profile in human papillary thyroid carcinomas (PTCs), using a microarray (miRNACHIP microarray), we have recently found an aberrant miR expression profile that clearly differentiates PTCs from normal thyroid tissues. It mainly consists in the overexpression of miR-221, miR-222, and miR-181b in PTCs. Functional studies,

performed by blocking the miR-221 function and over-expressing miR-221 in human PTC-derived cell lines, suggested a critical role of miR-221 overexpression in thyroid carcinogenesis. In fact, we found a significantly higher number of colonies in the thyroid carcinoma cells transfected with a miR-221 expression vector in comparison with the same cell line transfected with a backbone vector. Consistently, a significant reduction in cell growth was observed when the miR-221 function was blocked by antisense oligonucleotides (Pallante et al. 2006).

Using the algorithm of the bioinformatic programs miRGen (www.diana.pcbi.upenn.edu/miRGen; Megraw et al. 2007), TargetScan (Lewis et al. 2003), Pictar (Krek et al. 2005), and miRanda (John et al. 2004) to predict human miR gene targets, we identified the CDKN1B (p27^{Kip1}) gene as a putative target of miR-221 and miR-222. p27^{Kip1}, a member of the Cip/Kip family which also includes p21^{Cip1} and p57^{Kip2}, represents a very important regulator of cell cycle (Gu et al. 1993, Polyak et al. 1994, Chen et al. 1995). In fact, the Cip/Kip family together with INK4 proteins (p16^{INK4a}, p15^{INK4b}, p18^{INK4c}, and p19^{INK4d}), belongs to the cyclin-dependent kinase (CDK) inhibitors (Serrano et al. 1993, Guan et al. 1994, Hannon et al. 1994, Hirai et al. 1995). These proteins contrast the activities of CDKs which regulate the mitogen-dependent progression through the first gap phase (G₁) and initiation of DNA synthesis (S phase) during the mammalian cell division cycle (Kaldis 2007).

The p27^{Kip1} alterations have been frequently detected in human neoplasms. In fact, a reduced or absent p27^{Kip1} expression has been shown in the most aggressive ones (Slingerland & Pagano 2000). Moreover, in several cases, the impairment of the p27^{Kip1} function is due to a mislocalization of p27^{Kip1} from the nucleus to the cytoplasm induced by AKT activation (Viglietto et al. 2002). As far as thyroid neoplasias are concerned, 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 show an uncommon cytoplasmic localization of p27^{Kip1} protein, associated with a high Cdk2 activity (Baldassarre et al. 1999).

Here, we demonstrate that miR-221 and miR-222 regulate the p27^{Kip1} protein levels. This effect was dependent on two target sites in the 3'-untranslated region (UTR) of the p27^{Kip1} gene. Moreover, the enforced expression of miR-221 stimulates the TPC-1 cells to overcome the G₁/S block. Therefore, our data indicate that miR-221 and miR-222, negatively regulating p27^{Kip1} protein expression, are able to regulate cell cycle.

Materials and methods

Cell lines and transfections

The human thyroid carcinoma cell line TPC-1 (Tanaka et al. 1987) and HeLa cells were grown in Dulbecco's modified Eagle's medium (Gibco Laboratories) containing 10% fetal bovine serum (Gibco Laboratories), glutamine (Gibco Laboratories), and ampicillin/streptomycin (Gibco Laboratories) in a 5% CO₂ atmosphere. For transfection assay, TPC-1 and HeLa cells were plated at a density of 2.5×10^5 cells per well, in six-well plates, with three replicate wells for each condition, and transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 2'-O-Me-221-GAAACCCAGCAGACAAUGUAGCUL oligonucleotide, 2'-O-Me-222-GAGACCCAGUAGCCAGAU-GUAGCUL, and 2'-O-Me-enhanced green fluorescent protein (eGFP)-AAGGCAAGCUGACCCUGAAGUL (as control) were used in the antisense experiments. All 2'-O-methyl oligonucleotides were synthesized by Fidelity Systems, Inc. (Gaithersburg, MD, USA) as described previously (Meister et al. 2004) and were used at 200 nM concentration. RNA oligonucleotides corresponding to pre-miR negative control (#AM17110, Ambion, Austin, TX, USA), pre-miR-221, and pre-miR-222 were used at 100 nM final concentration in the sense experiments.

Protein extraction, western blotting, and antibodies

The cells were scraped in ice-cold PBS, and, subsequently, lysed in ice-cold NP40 lysis buffer (0.5% NP40, 50 mM HEPES (pH 7), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5 mM Na₃VO₄, 0.5 mM phenylmethylsulfonyl fluoride, Complete inhibitor (Roche)). Proteins were analyzed on polyacrylamide gel, transferred onto nitrocellulose membranes (Bio-Rad), incubated with specific primary antibodies, and visualized using enhanced chemiluminescence (GE Healthcare, Piscataway, NJ, USA). The antibodies used in this work were: anti-p27^{Kip1} (sc-C-19, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), anti-vinculin (sc-7649, Santa Cruz Biotechnology Inc.), and anti-GAPDH (Calbiochem, San Diego, CA, USA).

RNA extraction and quantitative reverse transcription (qRT)-PCR

Total RNA isolation from human tissues and cells was performed with Trizol (Invitrogen) according to the manufacturer's instructions. RNA was extracted from fresh specimens after pulverizing the tumors in a stainless steel mortar and pestle which were chilled on

dry ice. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis. RT-PCR analysis was performed on a panel of PTC samples of human thyroid origin and on transfected cells by using AmpliTaq DNA Polymerase (Applied Biosystems, Foster City, CA, USA) and the *mirVana* qRT-PCR miRNA Detection Kit (Ambion) following the manufacturer's instructions. Reactions contained *mirVana* qRT-PCR Primer Sets (Ambion) specific for miR-221, miR-222, and U6 (used to normalize RNA levels). qRT-PCR for p27^{Kip1} mRNA was performed by using TaqMan Gene Expression Assay (Ambion).

Flow cytometric analysis

TPC-1 cells were transfected with miR-221 and the scrambled oligonucleotides, deprived of serum, and analyzed by flow cytometry after 48 h, as described. Briefly, cells were harvested in PBS containing 2 mmol/l EDTA, washed once with PBS, and fixed for 2 h in cold ethanol (70%). Fixed cells were washed once in PBS and treated with 40 µg/ml RNase A in PBS for 30 min. They were then washed once in PBS and stained with 50 µg/ml propidium iodide (Roche). Stained cells were analyzed with a fluorescence-activated cell sorter (FACS) Calibur (Becton-Dickinson, Franklin Lakes, NJ, USA), and the data were analyzed using a mod-fit cell cycle analysis program.

Plasmids and constructs

The 464 bp 3'-UTR region of p27^{Kip1} gene, including binding site for miR-221/222, was amplified from HEK293 cells by using the following primers:

p27 gene-3'-UTR-XbaI-Fw, 5'-AATTTCTAGAGCTGACTTCATGGAATGGAC-3' and p27 gene-3'-3'UTR-XbaI-Rev, 5'-AATTTCTAGACAC-CAGATCTCCCAATGAG-3'.

The amplified fragment was cloned into pGL3-Control firefly luciferase reporter vector (Promega) at the XbaI site.

Deletions into the miR-221/222-binding sites of the p27 gene 3'-UTR were introduced by using Quik-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. The primers used were:

p27 gene mut1-Fw, 5'-AAGCGTTGGATTATGCAATTAGGTTTTTCC-3'; p27 gene mut1-Rev, 5'-CCTAATTGCATAATCCAACGCTTTTAGAGGCAGATC-3'; p27 gene mut2-Fw, 5'-TTACCTTTTAGCCACATAAACTTTGGGGAAGGGAGGGCAGGGT-3'; and p27 gene mut2-Rev, 5'-AGTTTATGTGTCAAAGGTAAAACTATATACAGGTAGT-3'.

Transfection efficiency was corrected by a *Renilla* luciferase vector (pRL-CMV, Promega).

Luciferase target assays

TPC-1 and HeLa cells were co-transfected in 12-well plates with the modified firefly luciferase vector described above, the *Renilla* luciferase reporter plasmid and with the RNA oligonucleotides. Firefly and *Renilla* luciferase activities were measured 24 h after transfection with the Dual-Luciferase Reporter Assay System (Promega). Firefly activity was normalized to *Renilla* activity to control the transfection efficiency.

Results

The miR cluster 221/222 represses p27^{Kip1} expression

Our previous studies demonstrated that blockage of the miR-221 and miR-222 was able to inhibit the growth of a thyroid papillary carcinoma cell line, and that the growth of the same cells was stimulated by miR-221 and miR-222 overexpression (Pallante *et al.* 2006, Visone *et al.* unpublished data). These results suggested that the miRs might participate in or simply be associated with regulatory events involved in the modulation of gene products having a role in cell growth regulation. Using bioinformatic tools (miRGen, TargetScan, Pictar, and miRanda) to search for potential mRNA targets of human miR-221 and miR-222, we identified several genes as being potentially targeted by these miRs. Among them, we selected the CDKN1B (CDK inhibitor 1B (p27^{Kip1}, Kip1) on its acknowledged role in cell cycle regulation. This choice was also dependent on previous results showing that miR-221 and miR-222 play an important role in the positive regulation of thyroid cell growth (Pallante *et al.* 2006), and that reduced p27 protein levels were detected in a significant number of human thyroid carcinomas in the absence of significant changes in p27 mRNA levels (Baldassarre *et al.* 1999).

Two sites in the 3'-UTR of the *CDKN1B* gene that match the miR-221 and miR-222 seed sequences were predicted (Fig. 1A). To validate the influence of miR-221/222 cluster on the p27^{Kip1} target, we transfected the miR-221 and/or miR-222, or their inhibitors, as 2'-O-Me-221 and/or 2'-O-Me-222, into the TPC-1 thyroid papillary carcinoma cell line and HeLa cells, and we searched for changes in p27^{Kip1} protein levels by western blot analysis. Introduction of both miR-221 and miR-222 decreased p27^{Kip1} protein levels (Fig. 1B). Conversely, the inhibitors,

Figure 1 $p27^{Kip1}$ protein is negatively regulated by miR-221/222 cluster. (A) Schematic representation of $p27^{Kip1}$ gene 3'-UTR target sites for miR-221 and miR-222. (B) Immunoblots for $p27^{Kip1}$ and vinculin protein as loading control. The proteins were extracted from the untransfected and miR-221/222 sense- or antisense-transfected TPC-1 and HeLa cells 48 h after transfection. (C) qRT-PCR analysis of $p27^{Kip1}$ mRNA in the same samples shown in (B). The fold change values indicate the relative change in $p27^{Kip1}$ expression levels between scrambled-treated and miR-treated cells, normalized with GAPDH.

2'-O-Me-221 and 2'-O-Me-222, increased $p27^{Kip1}$ protein amounts. No significant additive effects were observed when the same cells were transfected with both the miRs or their inhibitors (Fig. 1B). Interestingly, no significant changes in the $p27^{Kip1}$ mRNA levels were observed in the cells either transfected with the miR-221 and miR-222 or their inhibitors (Fig. 1C). This result validates a post-transcriptional regulation of the $p27^{Kip1}$ protein by miR-221 and miR-222, and also excludes their role in $p27^{Kip1}$ mRNA degradation.

Most miRs are thought to control gene expression by base-pairing with the miR-recognizing elements (miR-RE) found in their messenger target. To demonstrate that the direct interaction between the miR-221/222 and the CDKN1B mRNA was responsible for decreased expression of the $p27^{Kip1}$, we inserted downstream of the luciferase ORF the 464 bp (1159–1623) of the 3'-UTR of the CDKN1B mRNA. This reporter vector was transfected into TPC-1 and HeLa cells with i) the miR-221 and/or miR-222 oligonucleotide precursors, ii) the 2'-O-Me-221 and/or

2'-O-Me-222 and iii) a control not targeting scrambled oligonucleotide. The luciferase activity was markedly diminished after miR-221 and miR-222 transfection when compared with the scrambled oligonucleotide (Fig. 2B). Conversely, an increase in the luciferase activity was observed after transfection with the miR-221 and miR-222 inhibitors (Fig. 2B). These results indicate that both the miRs interfere with CDKN1B translation via direct interaction with the 3'-UTR. This conclusion is further supported by similar experiments in which we used as a reporter construct the same vector of the previous experiments, but carrying target sites modified by introducing point deletion in one or both sites together (deletion of eight nucleotides in the targeting site 1 or deletion of four nucleotides in the targeting site 2; Fig. 2A). Only the reporter vector carrying deletion in both target sites was insensitive to the effect of miR-221 and miR-222 (Fig. 2C) proving that the modification in only one target site of CDKN1B 3'-UTR is not enough to block the function of the miR-221/222 cluster.

Figure 2 The 3'-UTR of *p27^{Kip1}* gene enables miR-221 and miR-222 regulation. (A) Alignment of the miR-221/222 cluster with the insert derived from *p27^{Kip1}* gene 3'-UTR. Note the complementarity at the 5'-end of the miR-221/222 cluster where the 'seed' region is located. The inserts were cloned into the pGL3 control vector (pLuc *p27^{Kip1}* gene 3'-UTR). Three types of 3'-UTR mutants were constructed: a without all the nucleotides of the 'seed' region of miR::mRNA interaction (Site 1, pLuc *p27^{Kip1}* gene 3'-UTR Mut 1), b with a deletion of 3 bp in the 'seed' region of site 2 (Site 2, pLuc *p27^{Kip1}* gene 3'-UTR Mut 2), and c with modifications in both the target sites (pLuc *p27^{Kip1}* gene 3'-UTR Mut 1+2). (B) Relative luciferase activity in TPC-1 and HeLa cells transiently transfected with miR-221/222 oligonucleotides (sense and antisense) and a control not targeting scrambled oligonucleotide. The relative activity of firefly luciferase expression was standardized to a transfection control using *Renilla* luciferase. The results are reported as the mean of expression values with error bars indicating s.d. (mean $\pm$ s.d.); $n=6$. (C) Relative luciferase activity in TPC-1 cells transfected with miR-221/222 antisense oligonucleotides. The results are reported as the mean of the expression values with error bars indicating s.d. (mean $\pm$ s.d.); $n=6$.

miR-221 and miR-222 regulate cell cycle

Since the *p27^{Kip1}* protein has a key role in the cell cycle, particularly in the cell growth arrest at the G1/S transition, we have analyzed the possible role of miR-221 as a candidate cell cycle regulator. Therefore, the TPC-1 cells have been transfected either with miR-221 or with the scrambled control oligonucleotide,

deprived of serum, and then assayed by flow cytometric analysis after 48 and 72 h of starvation. A significant increase in the transition from the G1 to the S phase was observed in miR-221-treated cells when compared with scrambled-treated cells at 48 h post-starvation (Fig. 3A). In fact, as far as the scrambled-treated TPC-1 cells are concerned, 67.41% of the cells were counted in G0/G1 and 3.37% in the S phase, whereas the miR-221-treated cells showed 56.70% of the cells in G0/G1 and 19.77% in S phase. Western blot confirmed the reduction in *p27^{Kip1}* protein level in miR-221-treated TPC-1 cells (Fig. 3B). Similar results were obtained when the TPC-1 cells were transfected with the miR-222 oligonucleotide (data not shown).

miR-221 and miR-222 likely regulate *p27^{Kip1}* protein also *in vivo*

Previous works have constantly shown a reduction in *p27^{Kip1}* protein levels in human PTCs using different technologies such as immunohistochemistry and western blotting (Baldassarre *et al.* 1999, Tallini *et al.* 1999, Saltman *et al.* 2006). Moreover, two recent reports have evidenced a drastic increase in the miR-221 and miR-222 in the majority of PTCs (He *et al.* 2005, Pallante *et al.* 2006). These results lead to the hypothesis that the increase in the miR-221 and miR-222 might, at least partially, account for the *p27^{Kip1}*-reduced levels in PTCs. To verify this hypothesis, we have analyzed the *p27^{Kip1}* and the miR-221 and miR-222 levels in a set of PTCs. As shown in the Fig. 4A, decreased *p27^{Kip1}* protein levels were observed in 8 out of 11 PTCs, most of them showing an increase in miR-221 and miR-222 (Fig. 4B)

Figure 3 Flow cytometric analysis of TPC-1 cells transfected with the miR-221 or the scrambled oligonucleotide. (A) The TPC-1 cells were transfected with the scrambled or the miR-221 oligonucleotide. Then, the cells were deprived of serum, and after 48 h the DNA of the transfected TPC-1 cells was analyzed by flow cytometry after propidium iodine staining. (B) Immunoblots for *p27^{Kip1}* protein and vinculin as loading control.

Figure 4 p27^{Kip1} protein expression is inversely correlated with miR-221 and miR-222 expression in PTC patients. (A) Western blot analysis of p27^{Kip1} protein expression. The level of GAPDH has been used as loading control. NT, normal thyroid tissue; PTC, papillary thyroid carcinoma. (B) miR-221 and miR-222 *miRvana* qRT-PCR analysis. The fold change values indicate the relative change in the expression levels between normal and carcinoma samples, assuming that the value of each matched normal thyroid tissue is equal to 1. (C) qRT-PCR analysis of p27^{Kip1} mRNA expression in the same PTC samples of (A) and (B). The fold change values indicate the relative change in the expression levels between carcinoma and normal tissues, assuming that the value of each matched normal thyroid tissue is equal to 1.

expression in the absence of any significant changes in the CDKN1B mRNA levels (Fig. 4C). Interestingly, the PTC samples, such as PTC 10, 26, and 56, showing a drastic reduction in p27^{Kip1} protein expression, present the highest miR-221 and miR-222 levels. Therefore, these results suggest that the miR-221 and miR-222 overexpression may have a role in the decreased expression of the p27^{Kip1} protein in PTCs.

Discussion

The CDK inhibitor p27^{Kip1} has been shown to have a critical role in the control of mammalian cell proliferation. In fact, p27^{Kip1} negatively regulates the action of CDKs that are necessary for DNA replication. The levels of p27^{Kip1} are high in quiescent cells, but following growth stimulation by mitogenic stimuli, p27^{Kip1} is degraded allowing CDKs to drive cells into S phase. The negative role of p27^{Kip1} in cell cycle progression, and then its putative tumor suppressor role in human cancer, is validated by the impairment of the p27^{Kip1} function in many types of human cancer, which correlates with tumor aggressiveness and poor prognosis, and with the high

rate-frequency of several types of benign and malignant neoplasias developing in mice null for the p27^{Kip1} gene (Fero *et al.* 1996). The regulation of the p27^{Kip1} expression and function essentially occurs at the post-transcriptional level; p27^{Kip1} degradation depends on the phosphorylation on the threonine residue 187 (T187). It has been seen to occur in cells in response to growth factor signaling. In fact, members of the mitogen-activated family of ERK kinases and also cyclin E-activated cdk2 have been implicated in p27^{Kip1} phosphorylation and its subsequent degradation. Moreover, the p27^{Kip1} function is regulated by AKT activity since it has been shown that AKT is able to phosphorylate T157, which maps within the nuclear localization signal of p27^{Kip1} causing retention of p27^{Kip1} in the cytoplasm, precluding p27^{Kip1}-induced G1 arrest. Such a mechanism of p27^{Kip1} impairment has been well described in breast carcinomas (Liang *et al.* 2002, Shin *et al.* 2002, Viglietto *et al.* 2002).

Here, we report a novel mechanism regulating p27^{Kip1} protein levels that is based on the over-expression of the miR-221 and miR-222, previously described in PTCs (He *et al.* 2005, Pallante *et al.* 2006). These miRs have a matched sequence in the 3'-UTR of the p27^{Kip1} gene, and may regulate the specific p27^{Kip1} mRNA translation, and then p27^{Kip1} protein levels. In fact, we show that the enforced expression of the miR-221 and miR-222 significantly decreased the p27^{Kip1} protein levels. This effect seems due to an inhibition of the p27^{Kip1} mRNA translation process since no significant changes were observed in the mRNA levels after miR-221 and miR-222 treatments. Consistent with this result, the transfection with the miR-221 and miR-222 inhibitors leads to a significant increase in the p27^{Kip1} protein level. Moreover, this result is shown by us in two different cell lines: the PTC-derived TPC-1 cells and the HeLa cells. The choice of the TPC-1 cell line for these studies was dependent on the presence of a significant amount of the p27^{Kip1} at nuclear level, then keeping its role on the cell cycle regulation, in this cell line, whereas p27^{Kip1} was detected only in the cytoplasm of the other thyroid carcinoma cell lines, then unable to block the transition G1/S (Baldassarre *et al.* 1999). We showed that miR-221 and miR-222 directly regulate the p27^{Kip1} mRNA translation since they negatively regulated the expression of a p27^{Kip1} gene 3'-UTR-based reporter construct and this regulation is dependent on two target sites located in the p27^{Kip1} gene 3'-UTR. In fact, the mutations in both these sites make the reporter construct insensitive to the miR-221 and miR-222 expression. However, it cannot be excluded that miR-221/222 could be also indirectly involved in the regulation of the p27^{Kip1}

protein levels by targeting the genes coding for proteins involved in p27^{Kip1} protein degradation.

The role of the miR-221/222 cluster in p27^{Kip1} protein regulation has an important implication since it can account for the reduced p27^{Kip1} expression in several tumors. In fact, overexpression of these miRs has been already described in PTCs and other tumors (He *et al.* 2005, Pallante *et al.* 2006, Volinia *et al.* 2006, Lee *et al.* 2007). According to this hypothesis, we show a significant inverse correlation between p27^{Kip1} protein levels and miR-221 and miR-222 expression in PTCs. This represents another example of miRs with potential oncogenic properties that act through the repression of a tumor suppressor gene (Calin & Croce 2006, Meng *et al.* 2006). Furthermore, the results shown here indicate that miR-221 and miR-222 play an important role in cell cycle regulation. In fact, miR-221 overexpression drives TPC-1 cells to the S phase overcoming the block in G1 under serum-free conditions.

We would like to point out that p27 is not the only target for miR-221 and miR-222. It has been previously shown (He *et al.* 2005) that c-Kit is a target of these miRs and consistently, they show that c-Kit expression correlates in most of the PTCs with miR-221/222 overexpression. However, it remains still to be defined the role of the loss of c-Kit expression in thyroid cell proliferation and carcinogenesis. On the basis of the Bioinformatic Analysis, miR-221 and miR-222 may regulate other target genes that still need to be identified and biologically validated, with a critical role in cell proliferation. Therefore, miR-221/222 overexpression likely has a key role in the process of thyroid carcinogenesis. This hypothesis is further supported by the finding that an increased miR-221 and miR-222 expression was observed in the apparently normal thyroid tissues adjacent to the PTC lesions (He *et al.* 2005). The generation and characterization of transgenic mice overexpressing miR-221 and miR-222, in progress in our laboratory, should give the appropriate answer to this question.

While this manuscript was submitted for publication, a paper demonstrating that the p27^{Kip1} is a target of miR-221 and miR-222 has been published. The authors show that the miR-221/222 expression is able to reduce the p27^{Kip1} protein level and modify the growth potential of prostate carcinoma cells by inducing a G1 to S shift in the cell cycle, and enhance their colony forming potential (Galardi *et al.* 2007). These data appear completely consistent with the results shown by us here and previously in thyroid cancer (Pallante *et al.* 2006).

It is also noteworthy to observe that the critical functions of the miR-221/222 cluster, reported here, may also open new therapeutic perspectives. In fact,

new innovative therapeutic approaches may be based on the restoration of the normal miR-221 and miR-222 levels in the cancers overexpressing them by the administration of synthetic antisense oligonucleotides, complementary to mature endogenous miRs.

In conclusion, taken together, the results shown here indicate a novel mechanism of regulation of the p27^{Kip1} protein levels, and then of the cell cycle, mediated by miR-221 and miR-222 overexpression.

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References

- Ambros V 2004 The functions of animal microRNAs. *Nature* **431** 350–355.
- Baldassarre G, Belletti B, Bruni P, Boccia A, Trapasso F, Pentimalli F, Barone MV, Chiappetta G, Vento MT, Spiezia S *et al.* 1999 Overexpressed cyclin D3 contributes to retaining the growth inhibitor p27 in the cytoplasm of thyroid tumor cells. *Journal of Clinical Investigation* **104** 865–874.
- Bartel DP 2004 MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116** 281–297.
- Calin GA & Croce CM 2006 MicroRNA signatures in human cancers. *Nature Reviews. Cancer* **6** 857–866.
- Chen J, Jackson PK, Kirschner MW & Dutta A 1995 Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374** 386–388.
- Fero ML, Rivkin M, Tasch M, Porter P, Carow CE, Firpo E, Polyak K, Tsai LH, Broudy V, Perlmutter RM *et al.* 1996 A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* **85** 733–744.
- Galardi S, Mercatelli N, Giorda E, Massalini S, Frajese GV, Ciafre SA & Farace MG 2007 miR-221 and miR-222 expression affects the proliferation potential of human prostate carcinoma cell lines by targeting p27kip1. *Journal of Biological Chemistry* **282** 23716–23724.
- Gu Y, Turck CW & Morgan DO 1993 Inhibition of CDK2 activity *in vivo* by an associated 20K regulatory subunit. *Nature* **366** 707–710.
- Guan KL, Jenkins CW, Li Y, Nichols MA, Wu X, O'Keefe CL, Matera AG & Xiong Y 1994 Growth suppression by p18, a p16INK4/MTS1- and p14INK4B/MTS2-related CDK6 inhibitor, correlates with wild-type pRb function. *Genes and Development* **8** 2939–2952.

- Hannon GJ, Casso D & Beach D 1994 KAP: a dual specificity phosphatase that interacts with cyclin-dependent kinases. *PNAS* **91** 1731–1735.
- He H, Jazdzewski K, Li W, Liyanarachchi S, Nagy R, Volinia S, Calin GA, Liu CG, Franssila K, Suster S et al. 2005 The role of microRNA genes in papillary thyroid carcinoma. *PNAS* **102** 19075–19080.
- Hirai H, Roussel MF, Kato JY, Ashmun RA & Sherr CJ 1995 Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. *Molecular and Cellular Biology* **15** 2672–2681.
- John B, Enright AJ, Aravin A, Tuschl T, Sander C & Marks DS 2004 Human MicroRNA targets. *PLoS Biology* **2** e363.
- Kaldas P 2007 Another piece of the p27Kip1 puzzle. *Cell* **128** 241–244.
- Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M et al. 2005 Combinatorial microRNA target predictions. *Nature Genetics* **37** 495–500.
- Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, Morgan DL, Postier RG, Brackett DJ & Schmittgen TD 2007 Expression profiling identifies microRNA signature in pancreatic cancer. *International Journal of Cancer* **120** 1046–1054.
- Lewis BP, Shih IH, Jones-Rhoades MW, Bartel DP & Burge CB 2003 Prediction of mammalian microRNA targets. *Cell* **115** 787–798.
- Lewis BP, Burge CB & Bartel DP 2005 Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120** 15–20.
- Liang J, Zubovitz J, Petrocelli T, Kotchetkov R, Connor MK, Han K, Lee JH, Ciarallo S, Catzavelos C, Beniston R et al. 2002 PKB/Akt phosphorylates p27, impairs nuclear import of p27 and opposes p27-mediated G1 arrest. *Nature Medicine* **8** 1153–1160.
- Megraw M, Sethupathy P, Corda B & Hatzigeorgiou AG 2007 miRGen: a database for the study of animal microRNA genomic organization and function. *Nucleic Acids Research* **35** D149–D155.
- Meister G, Landthaler M, Dorsett Y & Tuschl T 2004 Sequence-specific inhibition of microRNA- and siRNA-induced RNA silencing. *RNA* **10** 544–550.
- Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, Jiang J, Schmittgen TD & Patel T 2006 Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. *Gastroenterology* **130** 2113–2129.
- Pallante P, Visone R, Ferracin M, Ferraro A, Berlingieri MT, Troncone G, Chiappetta G, Liu CG, Santoro M, Negrini M et al. 2006 MicroRNA deregulation in human thyroid papillary carcinomas. *Endocrine-Related Cancer* **13** 497–508.
- Polyak K, Lee MH, Erdjument-Bromage H, Koff A, Roberts JM, Tempst P & Massague J 1994 Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78** 59–66.
- Saltman B, Singh B, Hedvat CV, Wreesmann VB & Ghossein R 2006 Patterns of expression of cell cycle/apoptosis genes along the spectrum of thyroid carcinoma progression. *Surgery* **140** 899–906.
- Serrano M, Hannon GJ & Beach D 1993 A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* **366** 704–707.
- Shin I, Yakes FM, Rojo F, Shin NY, Bakin AV, Baselga J & Arteaga CL 2002 PKB/Akt mediates cell-cycle progression by phosphorylation of p27(Kip1) at threonine 157 and modulation of its cellular localization. *Nature Medicine* **8** 1145–1152.
- Slingerland J & Pagano M 2000 Regulation of the cdk inhibitor p27 and its deregulation in cancer. *Journal of Cell Physiology* **183** 10–17.
- Tallini G, Garcia-Rostan G, Herrero A, Zeltermann D, Viale G, Bosari S & Carcangiu ML 1999 Downregulation of p27KIP1 and Ki67/Mib1 labeling index support the classification of thyroid carcinoma into prognostically relevant categories. *American Journal of Surgical Pathology* **23** 678–685.
- Tanaka J, Ogura T, Sato H & Hatano M 1987 Establishment and biological characterization of an *in vitro* human cytomegalovirus latency model. *Virology* **161** 62–72.
- Viglietto G, Motti ML, Bruni P, Melillo RM, D'Alessio A, Califano D, Vinci F, Chiappetta G, Tschlis P, Bellacosa A et al. 2002 Cytoplasmic relocalization and inhibition of the cyclin-dependent kinase inhibitor p27(Kip1) by PKB/Akt-mediated phosphorylation in breast cancer. *Nature Medicine* **8** 1136–1144.
- Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, Visone R, Iorio M, Roldo C, Ferracin M et al. 2006 A microRNA expression signature of human solid tumors defines cancer gene targets. *PNAS* **103** 2257–2261.