

MicroRNA deregulation in human thyroid papillary carcinomas

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Abstract

MicroRNAs (miRNAs) are a class of small non-coding RNAs involved in a wide range of basic processes such as cell proliferation, development, apoptosis and stress response. It has recently been found that they are also abnormally expressed in many types of human cancer. We analyzed the genome-wide miRNA expression profile in human thyroid papillary carcinomas (PTCs) using a microarray (miRNACHIP microarray) containing hundreds of human precursor and mature miRNA oligonucleotide probes. Using this approach, we found an aberrant miRNA expression profile that clearly differentiates PTCs from normal thyroid tissues. In particular, a significant increase in miRNA (miR)-221, -222 and -181b was detected in PTCs in comparison with normal thyroid tissue. These results were further confirmed by northern blot and quantitative RT-PCR analyses. Moreover, RT-PCR revealed miR-221, -222 and -181b overexpression in fine needle aspiration biopsies corresponding to thyroid nodules, which were eventually diagnosed as papillary carcinomas after surgery. Finally, miR-221, -222 and -181b overexpression was also demonstrated in transformed rat thyroid cell lines and in mouse models of thyroid carcinogenesis. Functional studies, performed by blocking miR-221 function and by overexpressing miR-221 in human PTC-derived cell lines, suggest a critical role of miR-221 overexpression in thyroid carcinogenesis. In conclusion, these data, taken together, indicate an miRNA signature associated with PTCs, and suggest miRNA deregulation as an important event in thyroid cell transformation.

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Introduction

MicroRNAs (miRNAs) represent a class of endogenous, small, non-coding but functional RNAs of 19–23 nt cleaved from larger hairpin precursors (Bartel 2004). They comprise one of the most abundant classes of gene regulatory molecules in multicellular organism and likely influence the output of many protein-coding genes (Bartel 2004).

In fact, they are able to bind to the 3'-untranslated region (UTR) of target mRNAs and cause a block of translation or mRNA degradation (Rhoades *et al.* 2002, Tang *et al.* 2003, Ambros 2004, Bartel 2004). They are also functional when positioned in a coding region or even in 5'-UTR (Pillai 2005). The miRNA expression pattern is regulated during development and is generally tissue specific (Liu *et al.* 2004, Sempere *et al.* 2004). However, relatively

little is known about their possible functions in mammals.

At the present time, there are several reports indicating that miRNAs also represent a class of genes involved in human tumorigenesis, being aberrantly expressed, deleted, amplified or mutated in cancers (Calin *et al.* 2002, 2004a,b, 2005, McManus 2003, Michael *et al.* 2003, Metzler *et al.* 2004, Takamizawa *et al.* 2004, Eis *et al.* 2005, Gregory & Shiekhattar 2005, Johnson *et al.* 2005). Deregulated expression of certain miRNAs has been linked to human proliferative diseases such as B-cell chronic lymphocytic leukemia (Calin *et al.* 2002, Lagos-Quintana *et al.* 2003), breast (Iorio *et al.* 2005) and colorectal neoplasia (Michael *et al.* 2003), suggesting that they might play a role as oncogenes or tumor suppressors. Moreover, it has recently become possible to analyze the genome-wide miRNA expression thanks to the development of microarrays containing sequences corresponding to all known human miRNAs (Liu *et al.* 2004, Nelson *et al.* 2004, Liang *et al.* 2005). Therefore, these miRNA microarrays can allow the identification of miRNAs differentially expressed between normal and tumor samples.

Thyroid neoplasms represent a good model for studying the events involved in epithelial cell multi-step carcinogenesis, because they comprise a broad spectrum of lesions with different degrees of malignancy from benign adenomas, which are not invasive and very well differentiated, to the undifferentiated anaplastic thyroid carcinomas, which are very aggressive and always fatal. Papillary and follicular carcinomas, the most common forms of thyroid cancer, represent intermediate forms of neoplasia being differentiated and having a good prognosis (Hedinger *et al.* 1989, Wynford-Thomas 1997).

In this study, we have analyzed the genome-wide miRNA expression profile in 30 human papillary thyroid carcinoma (PTC) samples vs 10 normal thyroid tissue samples using a microarray containing oligonucleotide probes corresponding to 245 human precursors and mature miRNAs (miRNACHIP microarray; see Materials and methods). A subset of miRNAs was found to be overexpressed in PTC samples. In particular, miRNA (miR)-221, -222 and -181b were overexpressed in most of the PTCs analyzed, and also in fine needle aspiration biopsies (FNABs) originating from patients affected by PTC. Blocking miR-221 function by antisense methodology led to a reduced cell growth of a human PTC cell line, while its overexpression led

to increased colony formation, indicating a critical role of miR-221 overexpression in thyroid carcinogenesis.

Materials and methods

Thyroid tissue samples

Human neoplastic thyroid tissues and normal adjacent tissue or the controlateral normal thyroid lobe were obtained from surgical specimens and immediately frozen in liquid nitrogen. Thyroid tumors were collected at the Service d'Anatomopathologie, Centre Hospitalier Lyon Sud, Pierre Benite, France. RNA from 10 normal thyroid samples and 30 samples from patients with PTC were assessed for miRNA expression.

FNAB

The FNABs were performed at the Dipartimento di Anatomia Patologica e Citopatologia, Naples as described elsewhere (Zeppa *et al.* 1990, Troncone *et al.* 2000). Samples were obtained from eight patients with thyroid neoplasias who subsequently underwent surgery because examination of the FNAB yielded cytologic diagnoses suspicious for cancer. Normal thyroid cells, used as controls, were obtained from FNABs of thyroids carrying non-neoplastic nodules. FNAB samples were washed twice with $1 \times$ PBS and then processed for RNA extraction following the same procedure as that outlined below.

RNA extraction

Total RNA isolation was performed with Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA was extracted from fresh specimens after pulverizing the tumors in a stainless steel mortar and pestle that were chilled on dry ice. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

miRNACHIP microarray

Microarray experimental procedures were performed as previously described (Liu *et al.* 2004). Briefly, labeled targets from 5 μ g total RNA from each sample were biotin-labeled during reverse transcription using random examers. Hybridization was carried out on an miRNA microarray chip (KCI version 1.0 (Liu *et al.* 2004)) containing 368 probes in triplicate, corresponding to 245 human and mouse miRNA genes. Hybridization signals were

detected by biotin binding of a streptavidin-alexa 647 conjugate using a Perkin-Elmer ScanArray XL5K (Perkin-Elmer, Wellesley, MA, USA). Scanner images were quantified by the Quantarray software (Perkin-Elmer). Raw data were normalized and analyzed by GENESPRING software, version 7.2 (Silicon Genetics, Redwood City, CA, USA). Expression data were median centered by using the GENESPRING normalization option. Statistical comparisons were done with the GENESPRING ANOVA tool.

Northern blot analysis

This was carried out as described previously (Calin *et al.* 2002). RNA samples (10 µg each) were electrophoresed on 15% acrylamide, 7 mol/l urea Criterion precasted gels (Bio-Rad, Hercules, CA, USA) and transferred onto Hybond-N⁺ membrane (Amersham Biosciences, Piscataway, NJ, USA). Hybridization was performed at 37 °C in 7% SDS/0.2 mol/l Na₂PO₄ (pH 7.0) for 16 h. Membranes were washed at 42 °C, twice with 2 × standard saline phosphate (0.18 mol/l NaCl/10 mmol/l phosphate (pH 7.4)), 1 mmol/l EDTA (saline-sodium phosphate-EDTA; SSPE) and 0.1% SDS and twice with 0.5 × SSPE/0.1% SDS. The oligonucleotides used as probes, complementary to the sequences of the mature miRNAs, were as follows. miR-221-probe 5'-GAAACCCAGCAGACAATGTAGCT-3'; miR-222-probe 5'-GAGACCCAGTAGCCAGATGTAGCT-3'; miR-181b-probe 5'-CCCACCGACAGCAATGAATGTT-3'.

An oligonucleotide complementary to the U6 RNA (5'-GCAGGGGCCATGCTAATCTTCTCTGTATCG-3') was used to normalize expression levels. Totally 200 ng of each probe were end labeled with 100 mCi [γ -³²P]ATP using the polynucleotide kinase (Roche, Basel, Switzerland). Blots were stripped by boiling in 0.1% SDS for 10 min before re-hybridization and were successfully re-probed up to three times.

Quantitative RT-PCR for miRNA precursors

Quantitative RT-PCR was performed as described by Schmittgen *et al.* (2004). Briefly, RNA was reverse transcribed to cDNA with gene-specific primers and Thermoscript (Invitrogen), and the relative amount of each miRNA was normalized to the U6 RNA using the equation $2^{-\Delta C_T}$, where $\Delta C_T = (C_{TmiRNA} - C_{TU6RNA})$ (Schmittgen *et al.* 2004). PCRs were performed in triplicate using iCycler (Bio-Rad) with SYBR Green PCR Master Mix

(Applied Biosystems, Foster City, CA, USA) as follows: 95 °C for 10 min and 40 cycles (95 °C for 15 s and 60 °C for 1 min). A dissociation curve was run after each PCR in order to verify amplification specificity. The miRNAs analyzed included miR-221 and -222 and -181b-1 precursors. The primers used were as follows. Human miR-221 forward 5'-TTCGTTAGGCAACAGCTACATT-3', human miR-221 reverse 5'-GAACATGTTTCCAGGTAGCC-3'; human miR-222 forward 5'-GCTGCTGGAAGGTGTAGTA-3', human miR-222 reverse 5'-GATGCCATCAGAGACCCAGT-3'; human miR-181b-1 forward 5'-ATCAACATTCATTGCTGTCGG-3', human miR-181b-1 reverse 5'-ATTGTTCA GTGAGCTTGTTCCA-3'; rat miR-221 forward 5'-TTTGTAGGCAACAGCTACATT-3', rat miR-221 reverse 5'-AGAAATGCTTCCAGGTAGCC-3'; rat miR-181b-1 forward 5'-ATCAACATTCATTGCTGTCGG-3', rat miR-181b-1 reverse 5'-ATTGTTCA GTGAGCTTTTCTA-3'; mouse miR-221 forward 5'-TTTGTAGGCAACAGCTAC-3', mouse miR-221 reverse 5'-TTCCAGGTAGCCTGA AAC-3'; mouse miR-181b-1 forward 5'-AACATTCAACGCTGTCGGT-3', mouse miR-181b-1 reverse 5'-TTGCATTCATTGTTCA GTGAG-3'. Finally, primers for human, rat and mouse U6 were 5'-CTCGCTTCGGCAGCACA-3' for forward and 5'-AACGCTTCACGAATTTGCGT-3' for reverse.

Cell culture

The human thyroid carcinoma cell lines TPC-1 (Tanaka *et al.* 1987), NPA (Pang *et al.* 1989), B-CPAP (Fabien *et al.* 1994) and FB-2 (Basolo *et al.* 2002) were grown in Dulbecco's modified Eagles' medium (Gibco Laboratories, Carlsbad, CA, USA) containing 10% fetal calf serum (Gibco Laboratories), glutamine (Gibco Laboratories) and ampicillin/streptomycin (Gibco Laboratories) in a 5% CO₂ atmosphere. PC CL 3 cell line derived from Fischer rat thyroid (Fusco *et al.* 1987) was cultured in modified F12 medium supplemented with 5% calf serum (Gibco Laboratories) and six growth factors (thyrotropic hormone, hydrocortisone, insulin, transferrin, somatostatin and glycyl-histidyl-lysine (Sigma, St Louis, MO, USA)). PC CL 3 infected with several oncogenes PC v-ras-Ki, PC v-ras-Ha, PC v-raf, PC v-mos (Fusco *et al.* 1987), PC PyMLV (Berlingieri *et al.* 1988), PC E1A-raf (Berlingieri *et al.* 1993) and PC RET/PTC (Santoro *et al.* 1993) were cultured in the same medium as PC CL 3 but in the absence of the six growth factors.

Transfection assay

2'-O-Me-221-GAAACCCAGCAGACAAUGUAG-CUL oligonucleotide and 2'-O-Me-enhanced green fluorescent protein (eGFP)-AAGGCAAGCU-GACCCUGAAGUL (as control) were used in the antisense experiments. All 2'-O-methyl oligonucleotides were synthesized by Fidelity Systems, Inc. (Gaithersburg, MD, USA) as previously described (Meister *et al.* 2004) and were used at 200 nM concentration. NPA cells were plated at 1×10^5 cells per well, in six-well plates, with three replicate wells for each condition, transfected with siPORT neoFX (Ambion, Austin, TX, USA) according to the manufacturer's protocols, and counted by a Vi-Cell (Beckman Coulter, Inc., Fullerton, CA, USA) at 24, 48, 72 and 96 h post-transfection.

Plasmid constructs and cell colony-forming assay

miR-221 expression plasmid was constructed by cloning in sense orientation a genomic sequence including miR-221 in *Bgl*II/*Hind*III cloning sites of a

mammalian expression vector, pRS-GFP-Neo (OligoEngine, Seattle, WA, USA). Primers used were as follows: forward 5'-AAAGATCTCCCAGCATTTC-TGACTG-3' and reverse 5'-AAAAGCTTAGACCA-TTTGGGTGAAAT-3'. The expression of pRS-GFP-Neo-221 was assessed by northern blot as described, and western blotting for the GFP levels was used to show the equal efficiency of transfection with the pRS-GFP-Neo and pRS-GFP-Neo-221 constructs. NPA cells, plated at a density of 90% in 100 mm dishes, were transfected with 5 µg pRS-GFP-Neo-221 or pRS-GFP-Neo. After 24 h the antibiotic geneticin (G418; Gibco) was added. Two weeks after the onset of drug selection, the cells were fixed and stained with crystal violet (0.1% crystal violet in 20% methanol).

Results

miRNA expression profile of PTC versus normal thyroid

We used a miRNACHIP microarray (Liu *et al.* 2004) to evaluate the miRNA expression profile of 30

Table 1 miRNAs differentially expressed between human PTCs and normal thyroid tissues

miRNA name	P	Chr. map	Normal thyroid			PTC			Fold change
			Median	Range		Median	Range		
			Normalized	Min.	Max.	Normalized	Min.	Max.	
miR-125b-1	0.0471	11q24.1	7.41	3.28	13.80	12.30	5.04	31.26	1.66
miR-148	0.0466	7p15	0.89	0.62	1.17	0.70	0.39	1.40	-1.27
miR-224	0.0415	Xq28	3.59	1.79	8.34	6.42	2.32	16.52	1.79
miR-125b-2	0.0364	21q11.2	5.52	2.86	9.07	8.59	4.61	23.25	1.56
let-7f-1	0.0299	9q22.2	0.96	0.72	1.34	0.79	0.56	1.19	-1.21
miR-202	0.0275	1q26.3	0.78	0.57	1.14	0.98	0.67	1.50	1.25
miR-196-2	0.0224	12q13	0.84	0.57	1.48	1.16	0.66	2.13	1.38
miR-034	0.0143	1p36.22	0.81	0.57	1.10	1.03	0.73	1.46	1.27
miR-221	0.0143	Xp11.3	4.91	1.82	14.17	17.97	1.03	137.63	3.66
miR-151	0.00935	8q24.3	0.99	0.67	1.50	0.69	0.47	1.07	-1.42
miR-015-b	0.00879	3q26.1	0.90	0.82	1.08	0.75	0.44	1.32	-1.20
miR-142-as	0.00535	17q23	0.90	0.71	1.41	0.62	0.36	0.99	-1.46
miR-220	0.00435	Xq25	1.16	0.95	1.54	2.72	0.59	17.58	2.35
miR-222	0.00256	Xp11.3	3.34	1.51	8.80	15.46	1.27	87.92	4.63
miR-125a	0.00207	19q13.4	2.01	1.10	2.84	3.39	1.72	6.15	1.69
miR-199a-1	0.00178	19p13.2	0.99	0.79	1.39	0.75	0.42	1.05	-1.32
miR-140-as	0.000948	16q22.1	1.05	0.83	1.35	0.79	0.54	1.28	-1.33
miR-213	0.000595	1q31.3-q32.1	7.18	3.58	9.66	15.30	10.28	32.74	2.13
miR-200a	0.00026	1p36.3	0.85	0.59	1.08	1.20	0.89	1.62	1.42
miR-181a	0.000247	9q33.1-34.13	2.26	1.42	3.22	4.21	1.89	10.21	1.87
miR-181c	^a 7.88E-05	19p13.3	1.13	0.84	1.60	1.97	0.89	4.84	1.74
miR-181b	^b 3.68E-05	1q31.2-q32.1	1.50	1.22	2.30	4.45	1.69	8.43	2.97

^aexponential annotation for 0.0000788

^bexponential annotation for 0.0000368

Chr. map, chromosomal map

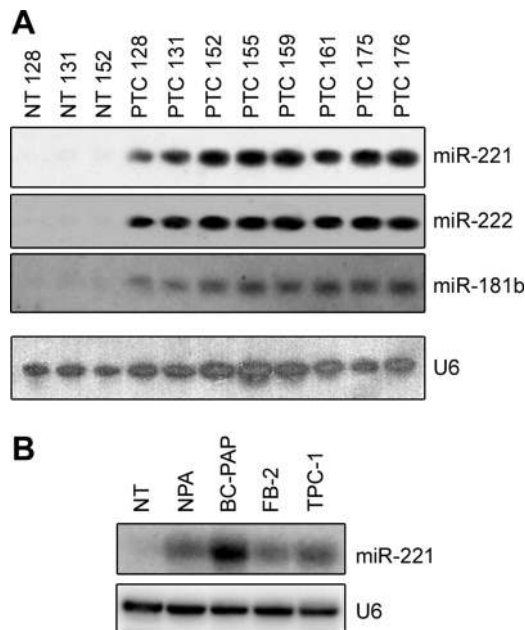


Figure 1 (A) Northern blot analysis performed on human PTC samples. The probe used corresponds to the complementary sequence of mature miR-221, -222 and -181b. The U6 probe was used for normalization of expression levels in the different samples. NT, normal thyroid tissues. (B) Northern blot analysis of miR-221 in a panel of four PTC cell lines of human origin.

PTCs and ten normal thyroid tissues. The normal thyroid samples were all matched with their corresponding carcinoma samples. By applying ANOVA analysis, we obtained a list of differentially expressed miRNAs ($P < 0.05$) between normal and neoplastic thyroids (Table 1). Altered level of the corresponding precursors were observed in some cases (data not shown).

Five miRNAs were overexpressed with a fold change equal to or higher than two in the neoplastic tissues vs the normal ones: they were miR-221, -222, -213, -220 and -181b. In contrast, none of the analyzed miRNAs showed a more than twofold reduction in its expression in the tumor samples. We then decided to concentrate on three of these miRNA for further studies since they showed the highest fold change. To confirm these results we performed northern blot analysis on a limited number of PTC samples using probes corresponding to miR-221, -222 and -181b (Fig. 1A). Northern blots confirmed the results obtained by microarray analysis, and in many cases the differences in miRNA expression between normal and neoplastic thyroid samples were even stronger than those anticipated by the microarray screen. In fact, as shown in Fig. 1A, miR-221, -222 and -181b were abundantly

expressed in all the eight PTCs examined, whereas their expression was only weakly detectable in the normal thyroid tissues. Northern blot analysis of four cell lines deriving from human PTC also revealed a higher miR-221 expression in comparison with the normal thyroid tissue (Fig. 1B). Next, we used quantitative RT-PCR to evaluate the expression of miR-221, -222 and -181b-1 precursors in a panel of eight thyroid follicular adenomas (FAs) and 39 PTCs, different from those analyzed by miRNACHIP microarray. Figure 2 shows that miR-221, -222 and -181b-1 precursors were overexpressed in almost all the carcinoma samples, being very high in some of them (miR-221 fold change: up to 74-fold with an average of 13.5; miR-222 fold change: up to 60-fold with an average of 13.7; miR-181b-1 fold change: up to 58.8-fold with an average of 13.48). These results confirmed that the increase in miR-221, -222 and -181b expression level represents a signature of the human PTC.

Interestingly, no miR-221 and -222 precursor overexpression was observed in the FA samples analyzed. In contrast, as shown in Fig. 2C, a modest increase in miR-181b-1 precursor expression was also detected in these FA samples (miR-181b-1 fold change: up to 8.56 with an average of 2.26).

Analysis of miRNA expression in thyroid FNABs

FNAB may be a useful tool in the pre-operative diagnosis of thyroid neoplasias in cases in which the material is too low to set up an immunohistochemical assay. To evaluate the applicability of miR-221, -222 and -181b gene expression analysis to FNAB samples, we studied eight cases of PTC. Normal thyroid cells, used as controls, were obtained from FNABs of thyroids carrying non-neoplastic nodules. The cytological specimens were analyzed for miR-221, -222 and -181b expression by quantitative RT-PCR on their precursors. A much higher expression of these three miRNAs was detected in seven out of eight analyzed carcinoma samples with respect to the normal thyroid cells (Fig. 3).

Overexpression of miR-221 and -181b in experimental rat and mouse models of thyroid carcinogenesis

Rat thyroid differentiated follicular cells, PC Cl 3 cells, have been previously transformed with several viral and cellular oncogenes. Some of them,

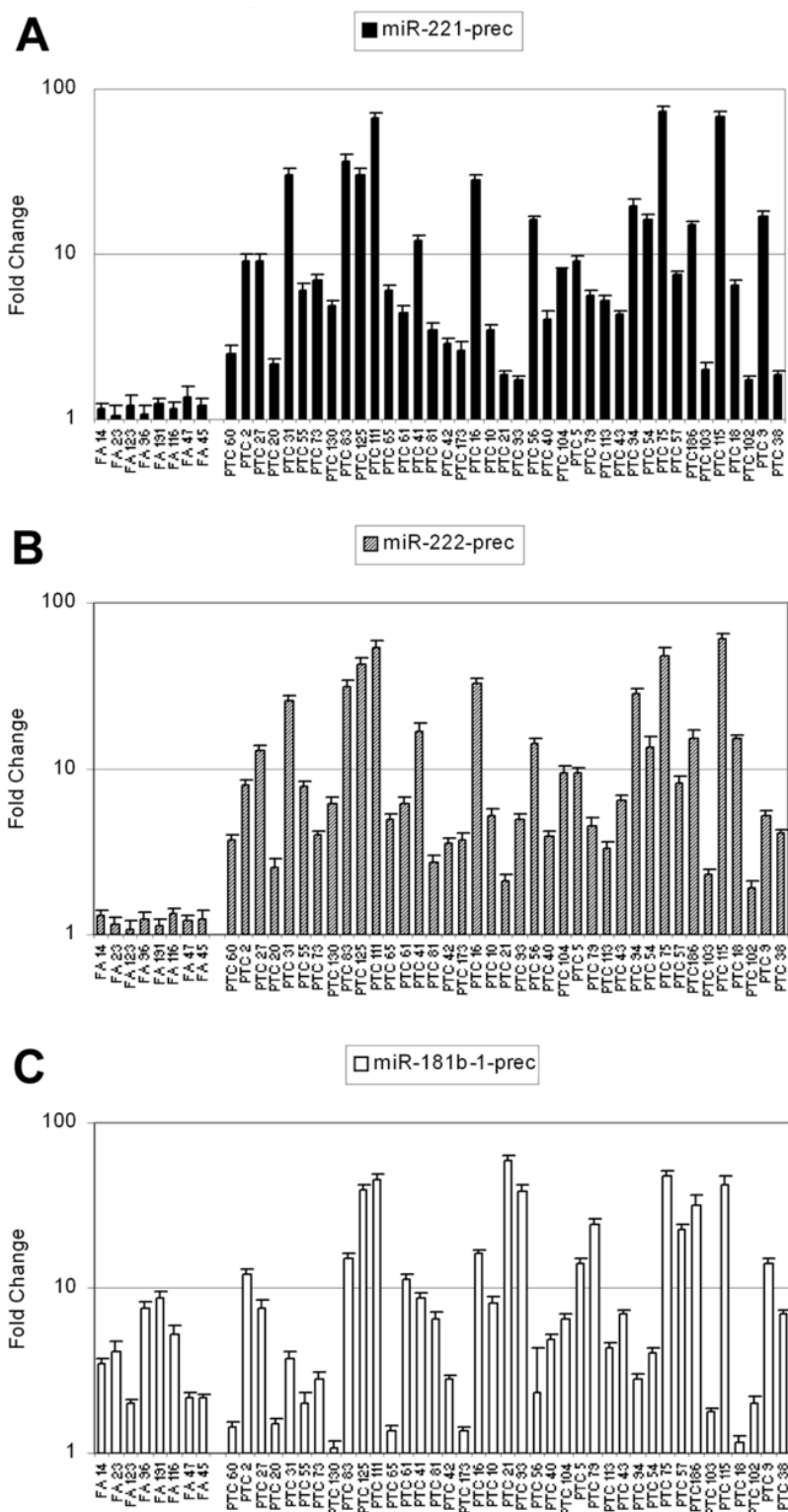


Figure 2 Quantitative RT-PCR analysis was performed on a panel of FAs and PTC samples of human thyroid origin. The fold change values indicate the relative change in the expression levels between tumor samples and the respective normal thyroid tissues deriving from each patient, assuming that the value of each normal sample was equal to 1. The expression of (A) miR-221, (B) -222 and (C) -181b-1 precursors (prec) are shown.

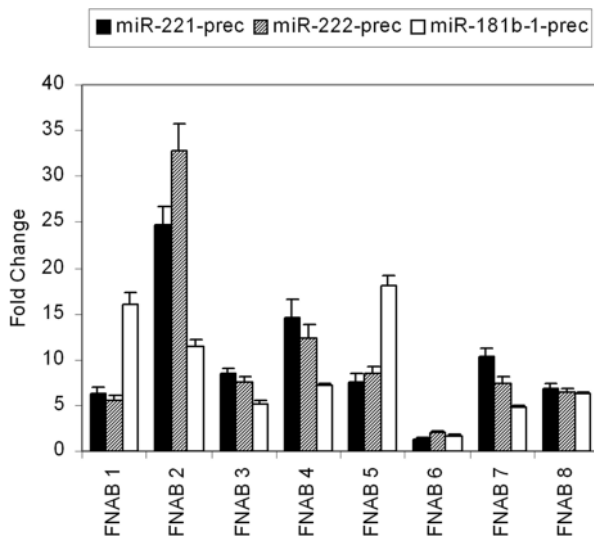


Figure 3 Quantitative RT-PCR analysis of miR-221, -222 and -181b-1 precursors (prec) was performed on human thyroid FNAB samples obtained from patients suspected to have a thyroid cancer and, subsequently, confirmed to have a PTC. Normal thyroid cells, obtained from FNAB of thyroids carrying non-neoplastic nodules, were used as controls. The fold change values indicate the relative change in the expression levels between tumor samples and the normal control, assuming that the value of the normal sample was equal to 1. FNAB 1-8, FNABs from different patients.

such as RET/PTC, v-raf and v-ras-Ha correspond to the oncogenes frequently activated in human PTCs. These cell lines were analyzed for the expression of miR-221 and -181b by quantitative RT-PCR on their precursors. Figure 4A shows that miR-221 and -181b-1 precursors were not detectable in the normal PC Cl 3 cells, whereas they were abundantly expressed in the PC Cl 3 cells transformed by v-ras-Ha, v-ras-Ki, v-raf, RET/PTC1, RET/PTC3, E1A-Abl, E1A-raf, PyPMLV and v-mos. The miR-221 expression was particularly high in the PC v-mos cell line. Northern blot, shown in Fig. 4B, essentially confirmed the miR-221 results obtained by quantitative RT-PCR. Similar results were obtained when miR-222 expression was evaluated (data not shown).

PTCs developing in transgenic animal lines expressing tyrosine kinase receptor TRK) (thyroglobulin (Tg)-TRK) (Russel *et al.* 2000), RET/PTC3 (Tg-RET/PTC3) (Powell *et al.* 1998) and HPV E7 (Tg-E7) (Ledent *et al.* 1995) oncogenes under the transcriptional control of the Tg promoter have also been analyzed for miR-221 and -181b-1 precursor expression by quantitative RT-PCR analysis. As shown in Fig. 4C, elevated specific miR-221 and -181b-1 precursor RNA levels were

observed in the thyroid carcinomas samples, whereas they were expressed at low levels in normal mouse thyroid tissues. Similar results were obtained for miR-222 (data not shown). Therefore, miR-221, -222 and -181b were also overexpressed in experimental models of thyroid carcinogenesis.

Overexpression of miR-221 affects the growth of thyroid cancer cell lines

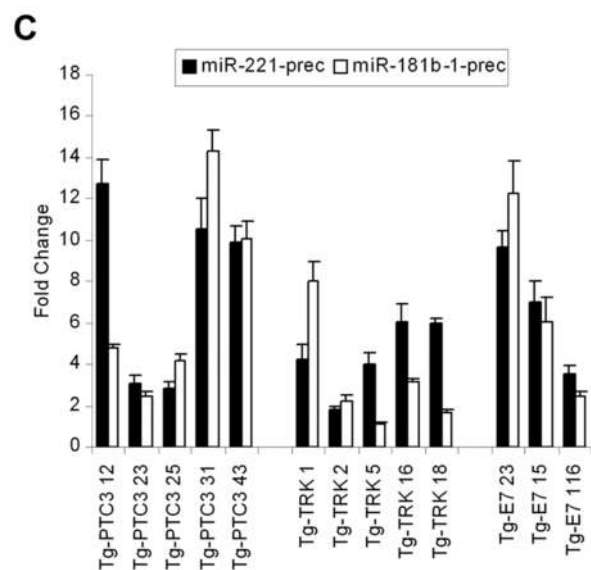
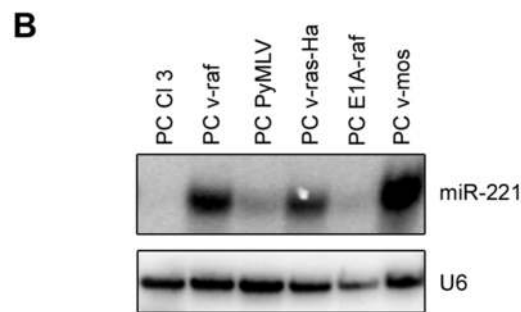
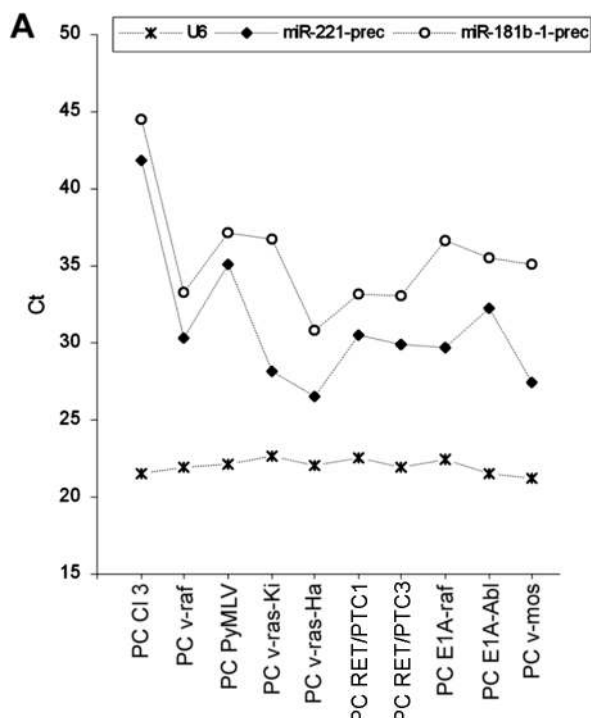
The aberrant expression of miR-221, -222 and -181b in PTCs prompted us to investigate whether their overexpression could be a causative event of the thyroid cell proliferation. To this purpose, we selected miR-221 for functional studies starting with a colony-forming assay on a human PTC cell line (NPA). As shown in Fig. 5A, transfection with miR-221 gave rise to a higher number (>twofold) of colonies in comparison with the same cells transfected with the empty vector. Northern blot analysis confirmed the overexpression of miR-221 in the NPA transfected cells (data not shown).

Blocking miR-221 expression inhibits thyroid carcinoma cell growth

In order to investigate the role of miR-221 overexpression in the growth of thyroid neoplastic cells, we blocked the miR-221 function by transfecting the NPA cells with modified antisense oligonucleotides for the miR-221. As shown in Fig. 5B, no miR-221 RNA was detected in the NPA cells treated with the antisense oligonucleotides (2'-O-Me-221), while it was present in those treated with the control oligonucleotides (2'-O-Me-eGFP). A significant reduction of NPA cell number treated with the antisense oligo compared with the control cells was observed at 72 and 96 h after oligonucleotide transfection (Fig. 5C). These results suggest that miR-221 overexpression has an important role in thyroid carcinoma cell proliferation.

Discussion

miRNAs mediate a recently recognized form of translational inhibition that alters the levels of critical proteins, thereby providing a mechanism for spatio-temporal control of developmental and homeostatic events across a wide range of plants and animals (Bartel 2004, Ambros 2004). Because abnormal proliferation and/or apoptosis are a hallmark of human cancers, it seems possible that miRNA expression patterns might denote the



malignant state. Indeed, altered expression of a few miRNAs has been found in some tumor types, and recent studies have shown that altered expression of specific miRNA genes contributes to the initiation and progression of cancer (Calin *et al.* 2002, 2004a,b, 2005, Lagos-Quintana *et al.* 2003, McManus 2003, Michael *et al.* 2003, Metzler *et al.* 2004, Takamizawa *et al.* 2004, Eis *et al.* 2005, Gregory & Shiekhattar 2005, Iorio *et al.* 2005, Johnson *et al.* 2005). Therefore, miRNA expression profiles offer the potential to inform cancer classification, diagnosis and prognosis.

Thyroid neoplasms represent an excellent model for studying the process of cell transformation since they include a broad spectrum of histotypes showing different degrees of malignancy (Hedinger *et al.* 1989, Wynford-Thomas 1997). In our study, we have analyzed, by miRNACHIP microarray, 30 PTCs and 10 normal thyroid samples to identify miRNAs whose expression is significantly deregulated in cancer. We have indeed identified about 30 miRNAs significantly deregulated and at least five of them whose expression was highly upregulated. These results leave few doubts that aberrant expression of miRNAs is common in human thyroid cancers; however, additional experiments are necessary to conclusively demonstrate that miRNA upregulation functions in human thyroid carcinogenesis.

Interestingly, we have found miRNAs significantly upregulated in PTC samples, whereas no miRNA has been found downregulated with a fold

Figure 4 miR-221 and -181b-1 precursor (prec) expression in experimental models of thyroid carcinogenesis. A panel of rat thyroid cell lines was tested for miR-221 and -181b-1 precursor expression. (A) Quantitative RT-PCR analysis: RNA transcription levels of U6 reference gene (internal control) and miR-221 and -181b-1 precursors are expressed as absolute Ct values (Ct represents the amplification cycle number in which fluorescence levels are first detected for a sample). The same amount of RNA for each sample was used as a template to amplify U6, miR-221 and 181b-1 genes. (B) Northern blot analysis using a probe corresponding to complementary sequences of the mature miR-221. The U6 probe was used for normalization of expression levels in the different samples. (C) Quantitative RT-PCR analysis performed on a panel of experimental mouse models of thyroid carcinogenesis. Normal mouse thyroid tissues were used as controls. The fold change values indicate the relative change in the expression levels between tumor samples and normal thyroid samples, assuming that the value of each normal sample was equal to 1. Tg-PTC3, Tg-TRK and Tg-E7 are transgenic mouse lines expressing respectively RET/PTC3, TRK and HPV E7 oncogenes under the transcriptional control of the Tg promoter.

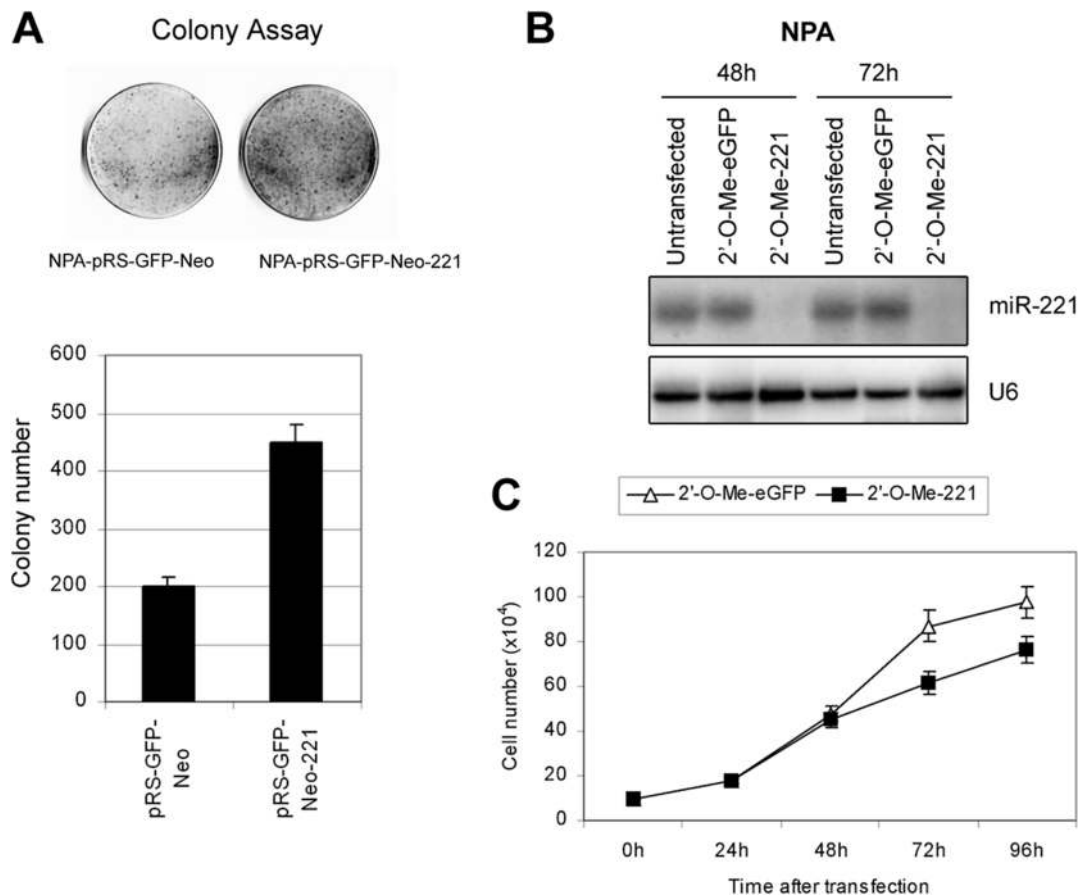


Figure 5 (A) Colony-forming assay experiment performed on NPA cells transfected with a vector expressing miR-221 (pRS-GFP-Neo-221) under the transcriptional control of the H1 gene promoter. The empty vector (pRS-GFP-Neo) was used as a control. The reported results are the mean of two experiments and error bars show s.d. (B) Northern blot assessing the effective downregulation of the endogenous miR-221 RNA, in NPA cells, after the transfection with 2'-O-methyl oligonucleotides corresponding to the complementary sequence of miR-221 (2'-O-Me-221). 2'-O-methyl oligonucleotides complementary to the sequence of GFP mRNA (2'-O-Me-eGFP) were used as a control. (C) Cells were counted at 24, 48, 72 and 96 h post-transfection of 2'-O-Me-221 and 2'-O-Me-eGFP oligonucleotides.

change higher than two. This would therefore suggest that gain- rather than loss-of-function of specific miRNAs is involved in PTC. Interestingly, preliminary data (not shown) on anaplastic thyroid carcinomas (ATCs) showed an opposite result: only a few miRNAs were upregulated (with a very low fold change), whereas several miRNAs were drastically downregulated. In ATCs it is possible that miRNAs might function as tumor suppressor or differentiation genes. This scenario shows some analogies with the other genetic lesions in thyroid carcinogenesis: activation of oncogenes (such as RET/PTC, TRK, B-raf, ras) in PTC, and loss-of-function of the p53 tumor suppressor in ATC.

miR-221, -222 and -181b induction is a constant feature of PTCs. Their upregulation therefore represents a real signature of PTC. Indeed, their

upregulation has been validated by northern blot and quantitative RT-PCR on a large number of samples and, as in the case of miR-221, the over-expression may reach up to a 70-fold increase.

It is worth noting that miR-221 and -222 (both precursors and mature products) in our system showed a very high similar expression pattern. This is consistent with the observation that miR-221 and -222 are clustered on chromosome X (Altuvia *et al.* 2005) showing, like some other clustered miRNA genes, high similarity in sequence (Bartel 2004) and perhaps they might be transcribed as polycistrons (Bartel 2004, Cullen 2004, Baskerville & Bartel 2005). Our studies *in vivo* and *in vitro* confirmed their upregulation during thyroid cell transformation. In fact, miR-221 expression is completely absent in the normal rat thyroid cell

line PC Cl 3 and in mouse normal thyroid tissues, while it is clearly detectable in the corresponding transformed cells and in PTCs developed in Tg-RET/PTC, Tg-TRK and Tg-E7 mice. The same also occurs for miR-181b. As far as the mechanism underlying miRNA overexpression in PTCs, we can exclude the possibility that gene amplification plays a major role. It is likely that other mechanisms account for the miRNA upregulation during the process of thyroid carcinogenesis.

It is worth noting that miR-221 and -222 are not overexpressed in benign and well-differentiated FAs. These results suggest the measurement of these miRNA levels for a differential diagnosis between benign and malignant thyroid neoplasms. The analysis of a larger number of samples will be required to ascertain the miRNA expression evaluation as a valid tool for the diagnosis of thyroid neoplasias.

One of the aims of our work has also been to investigate a possible role of miR-221 overexpression in thyroid carcinogenesis. To answer this question we have followed two experimental approaches. We have performed a colony assay on a human thyroid carcinoma cell line transfected with a vector overexpressing the miR-221 and found that the number of colonies was significantly higher in the cells overexpressing miR-221. The other approach was based on blocking the miR-221 function by antisense oligonucleotides: a significant reduction in the cell growth was observed. Both these experiments therefore point to a role of miR-221 in thyroid carcinoma cell growth. The generation of transgenic mice overexpressing miR-221 under the transcriptional control of a thyroid specific promoter, such as that of the Tg gene, should give the definitive answer to this question. Experiments aimed at validating a role of miR-181b overexpression in thyroid cell growth are also in progress in our laboratories.

miRNAs are known to regulate the expression of genes involved in the control of development, proliferation, apoptosis and stress response (He & Hannon 2004). In animal cells, single-stranded miRNAs bind to specific target mRNAs through partial complementarity predominantly in the 3'-UTR. The bound mRNAs either remain untranslated, resulting in a decrease of the corresponding protein product, or are degraded by the RNA interference effector complex resulting in a decreased number of transcripts (Bartel 2004). We therefore looked at the predicted targets of the most significantly upregulated miRNAs. We used the most

commonly used algorithms TARGETSCAN (Lewis *et al.* 2003), PICTAR (Krek *et al.* 2005) and MIRANDA (John *et al.* 2004) to predict human miRNA gene targets. TARGETSCAN (2003 version) and MIRANDA identified c-KIT as a putative target of miR-221 (also of miR-222 in TARGETSCAN), while PICTAR identified c-KIT as a target of miR-181b. Moreover, it has also been demonstrated that miR-221 and miR-222 are really able to downregulate the level of c-KIT protein (Felli *et al.* 2005). Our preliminary data, obtained by western blot and immunohistochemistry, seem to indicate that c-KIT protein levels are drastically reduced in PTCs, confirming data published by our group some years ago (Natali *et al.* 1995).

While this work was in progress, a paper on the expression profile of miRNAs in PTCs has been published (He *et al.* 2005). Our results are not only confirmatory of these published data, but they also add some more information about the overexpression of these miRNAs in experimental thyroid cell and animal systems and, more importantly, suggest a role of miR-221 in the proliferation of human thyroid carcinoma cells and thus, probably, in the process of thyroid carcinogenesis. On the other hand, our paper has also revealed by quantitative RT-PCR an overexpression in PTC of miR-181b which has not been described previously.

In conclusion, this study has indicated a miRNA signature associated to PTCs and suggests a critical role of miR-221 overexpression in thyroid carcinogenesis.

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