Deregulation of microRNA expression in follicular cell-derived human thyroid carcinomas

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Abstract

Carcinoma of the thyroid gland is an uncommon cancer, but one of the most frequent malignancies of the endocrine system. Most thyroid cancers are derived from the follicular cells. Follicular carcinoma is considered more malignant than papillary thyroid carcinoma (PTC), and anaplastic thyroid cancer (ATC) is one of the most lethal human cancers. Even though several genetic lesions have been already described in human thyroid cancer, particularly in the papillary histotype, the mechanisms underlying the development of these neoplasias are still far from being completely elucidated. Some years ago, several studies were undertaken to analyze the expression of microRNAs (miRNAs or miRs) in thyroid carcinoma to evaluate a possible role of their deregulation in the process of carcinogenesis. These studies showed an aberrant microRNA expression profile that distinguishes unequivocally among PTC, ATC, and normal thyroid tissue. Here, other than summarizing the current findings on microRNA expression in human thyroid carcinomas, we discuss the mechanisms by which microRNA deregulation may play a role in thyroid carcinogenesis, and the possible use of microRNA knowledge in the diagnosis and therapy of thyroid neoplasms.

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Introduction

The thyroid gland is composed of two distinct hormone-producing cell types: follicular cells and parafollicular C-cells. Follicular cells, present in the monolayer epithelium, are responsible for iodine uptake and thyroid hormone synthesis. C-cells are intrafollicular or parafollicular cells that are responsible for the production of the calcium-regulating hormone calcitonin. The majority of the thyroid tumors (more than 95%) are derived from the follicular cells,

while a minority (3%), called medullary thyroid carcinomas, are C-cell-derived carcinomas (Carcangiu *et al.* 1984, LiVolsi & Asa 1994, DeLellis *et al.* 2004, Kondo *et al.* 2006).

Thyroid tumors are of two types: benign and malignant. Benign tumors are principally represented by adenomas, while malignant tumors are, in most cases, carcinomas. Thyroid carcinomas are one of the most common malignancies of the endocrine system, with ~37 000 new cases diagnosed in the United States in 2008 (Jemal *et al.* 2008). Follicular cell-derived carcinomas are commonly divided into well-differentiated thyroid carcinoma (WDTC), poorly differentiated thyroid carcinoma (PDTC), and undifferentiated types depending on various histological and clinical factors (Carcangiu *et al.* 1984, LiVolsi & Asa 1994,

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DeLellis et al. 2004, Kondo et al. 2006). WDTCs include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC) types.

PTCs are characterized by classical papillary architecture and cells with typical nuclear alterations (ground-glass nuclei; DeLellis et al. 2004). Very often, PTCs are multifocal and tend to metastasize to the regional lymph nodes. FTC is a quite uncommon tumor, accounting for about 10% of all thyroid carcinomas. It may be of conventional or oncocytic (Hurthle cell) type (DeLellis et al. 2004). FTC is well differentiated, usually unifocal, encapsulated, with a tendency to metastasize via the vascular system to the bones and lungs. It is likely that FTCs can develop from pre-existing benign follicular adenomas (FTAs) or by directly bypassing the stage of adenoma. The differential diagnosis between the FTCs and the, far more frequent, FTAs is difficult, thus making FTCs of noticeable clinical importance.

Anaplastic thyroid cancers (ATCs) are very rare tumors estimated to comprise about 2–5% of thyroid malignancies. ATCs are highly undifferentiated, extremely aggressive and insensitive to conventional radiotherapy and chemotherapy (Ain 1999, Yau et al. 2008). ATCs may derive from the progression of pre-existing PTCs and FTCs. Such a hypothesis is validated by the presence, in some patients, of undifferentiated (anaplastic) areas that co-exist with more differentiated areas of papillary or follicular type (Ain 1999). As far as PDTCs are concerned, their morphology and behavior is intermediate between WDTC and undifferentiated thyroid carcinomas (Carcangiu et al. 1984, DeLellis et al. 2004).

Radiation exposure is the major risk factor for PTCs. Indeed, PTC incidence was verified among the atomic bomb survivors of Hiroshima and Nagasaki (Williams 2002). In the 1950s, patients treated with irradiation in the head and neck region for the cure of thymic hyperplasia or mycotic infections developed higher susceptibility to PTCs. Furthermore, after the nuclear reaction accident of Chernobyl in April 1986, children, mostly in Belarus and Ukraine, exhibited an increased PTC incidence (up to 100 times). It was also found that post-Chernobyl PTCs, often presenting a particular type of histology with dense cell structure (solid PTC variant), were more aggressive than those found in regions not exposed to radiations (Williams 2002).

Endemic goiter due to the lack of iodine represents one of the major risk factors for FTC development since the incidence of FTCs is higher in areas of iodine deficiency than in those with a diet rich in iodine (Harach et al. 2002, DeLellis et al. 2004).

Genetic factors other than the environmental ones may as well play a role in thyroid carcinogenesis. An idiopathic form of familial nonmedullary thyroid carcinoma accounts for 3.5–6.2% of thyroid cancers (Lupoli et al. 1999). Familial thyroid cancers associated with tumor syndromes caused by known germline mutations include familial polyposis coli, Cowden disease, and Werner syndrome (Lindor & Greene 1998). It has recently been shown that an important role to the onset of familial PTCs and FTCs can be attributed to the common variants located in the loci 13q22.33 and 14q13.3. Forkhead box E1 (also known as thyroid transcription factor 2, *TTF2*) is the gene nearest to the 9q22.33 locus, and NK2 homeobox 1 (also known as *TTF1*) is among the genes located at the 14q13.3 locus. These variants account for an increased risk of both PTCs and FTCs. Approximately 3.7% of individuals are homozygous for both variants, and their estimated risk of thyroid cancer is 5.7-fold greater than that of noncarriers (Gudmundsson et al. 2009).

Several oncogenes have been involved in thyroid carcinoma development (Table 1). In PTCs, non-overlapping mutations of genes involved in the activation pathway of mitogen-activated protein kinase (MAPK), such as *RET*, *TRK*, *RAS*, and *BRAF*, have been found in about 70% of the cases (Kimura et al. 2003, Soares et al. 2003, Frattini et al. 2004). Indeed, a fraction of about 30% of PTCs present a typical gene alteration consisting in the rearrangement of *RET* proto-oncogene (Santoro et al. 2004; Table 1). At least ten different types of *RET*/PTC rearrangements have been reported (Nikiforov 2002). The *RET*/PTC rearrangements consist in the fusion of tyrosine kinase (TK) domain of *RET* with other genes that provide to the chimeric gene the promoter and the 5' coding region. *RET*/PTC1 and *RET*/PTC3 are the most frequent isoforms found in PTCs. *RET*/PTC1 was generated by the fusion of the *RET* TK domain with the 5' terminal region of a new gene that was denominated *H4* (now *CCDC6*; Grieco et al. 1990), whereas in *RET*/PTC3, the TK domain of *RET* is fused to the *RFG* gene (also designated *Ele1/ARA70/Nco4*; Santoro et al. 1994).

The isolation of *RET*/PTC genes together with the existence, in fewer cases, of analogous rearrangements of the *TRK* gene, which encodes another protein with a TK function, confirms that TKs play an important role in PTC formation. Under normal conditions, *TRK* encodes a receptor for the nerve growth factor. *TRK* rearrangements and fusion genes very similar to *RET*/PTC rearrangements have been described in about 10% of PTC cases (Pierotti et al. 1995; Table 1).

Table 1 Genetic alterations of human thyroid carcinomas of follicular cell origin

Tumor histotype	Cell type	Etiological factors	Molecular lesion	Prevalence (%)	References
Papillary thyroid carcinoma	Follicular cell	Ionizing radiation	RET rearrangement	30	Santoro <i>et al.</i> (2004)
			NTRK1 rearrangement	10	Pierotti <i>et al.</i> (1995)
			BRAF mutation (V600E)	40	Kimura <i>et al.</i> (2003) and Soares <i>et al.</i> (2003)
Follicular thyroid adenoma	Follicular cell	Iodine deficiency	RAS mutation (codons 12, 13, and 61)	10–20	Namba <i>et al.</i> (1990), Ezzat <i>et al.</i> (1996) and Vasko <i>et al.</i> (2004)
			RAS mutation (codons 12, 13, and 61)	20–40	Suarez <i>et al.</i> (1990), Esapa <i>et al.</i> (1999), Basolo <i>et al.</i> (2000) and Motoi <i>et al.</i> (2000)
			PAX8/PPAR γ rearrangement	2–10	Dwight <i>et al.</i> (2003), French <i>et al.</i> (2003) and Nikiforova <i>et al.</i> (2003)
Follicular thyroid carcinoma	Follicular cell	Iodine deficiency	RAS mutation (codons 12, 13, and 61)	40–50	Suarez <i>et al.</i> (1990), Esapa <i>et al.</i> (1999), Basolo <i>et al.</i> (2000) and Motoi <i>et al.</i> (2000)
			PAX8/PPAR γ rearrangement	35	Dwight <i>et al.</i> (2003), French <i>et al.</i> (2003) and Nikiforova <i>et al.</i> (2003)
			PIK3CA mutation	8	Paes & Ringel (2008)
Poorly differentiated thyroid carcinoma	Follicular cell	Tumor progression	PTEN mutation	7	Paes & Ringel (2008)
			RET rearrangement	13	Santoro <i>et al.</i> (2002)
			BRAF mutation (V600E)	12–17	Garcia-Rostan <i>et al.</i> (2005), Hou <i>et al.</i> (2007) and Santarpia <i>et al.</i> (2008)
			RAS mutation (codons 12, 13, and 61)	46–55	Ito <i>et al.</i> (1992), Donghi <i>et al.</i> (1993) and Fagin <i>et al.</i> (1993)
			TP53 mutation	17–38	Garcia-Rostan <i>et al.</i> (2001) and Miyake <i>et al.</i> (2001)
			CTNNB1 mutation	25	Garcia-Rostan <i>et al.</i> (2001) and Miyake <i>et al.</i> (2001)
Anaplastic thyroid carcinoma	Follicular cell	Tumor progression	BRAF mutation (V600E)	25–29	Garcia-Rostan <i>et al.</i> (2005), Hou <i>et al.</i> (2007) and Santarpia <i>et al.</i> (2008)
			RAS mutation (codons 12, 13, and 61)	6–52	Ito <i>et al.</i> (1992), Donghi <i>et al.</i> (1993) and Fagin <i>et al.</i> (1993)
			TP53 mutation	67–88	Garcia-Rostan <i>et al.</i> (2001) and Miyake <i>et al.</i> (2001)
			CTNNB1 mutation	66	Paes & Ringel (2008)
			PIK3CA mutation	16	Paes & Ringel (2008)
			PTEN mutation	14	Paes & Ringel (2008)

In most PTC cases, a specific point mutation of *BRAF* is found. The *BRAF* T1799A mutation is the most common genetic change in PTCs and occurs in about 40% of PTCs (Table 1; Kimura et al. 2003, Soares et al. 2003). As in melanomas, the most common *BRAF* mutation results from T>A transversions at position 1799, resulting in a valine> glutamate substitution at residue 600 (V600E) that destabilizes the inactive conformation of the RAF kinase (Dhillon & Kolch 2004).

In PTCs, *BRAF* mutation and *RET/PTC* rearrangements are mutually exclusive and cannot be found simultaneously in the same patients, yet they are not completely equivalent, since it has been shown that PTCs positive for *BRAF* are more aggressive than those positive for *RET/PTC* (Kimura et al. 2003, Soares et al. 2003).

It has recently been demonstrated that some types of PTCs developed after the Chernobyl nuclear accident present a different *BRAF* alteration since they harbored a rearrangement similar to the *RET/PTC* rearrangements, which results in the fusion between *BRAF* kinase domain and the *AKAP9* gene and is caused by a paracentric inversion of the long arm of chromosome 7 (Ciampi et al. 2005).

Mutations of the *RAS* genes (*K-RAS*, *H-RAS*, and *N-RAS*), present in codons 12, 13, and 61 (Table 1), are observed in 10–20% of PTCs (Namba et al. 1990, Ezzat et al. 1996, Vasko et al. 2004; Table 1). *RAS* gene mutations represent early molecular lesions since they are also frequently found in FTAs which are considered FTC precursors. In fact, *RAS* mutations are observed in 40–50% of conventional FTCs and in 20–40% of FTAs (Suarez et al. 1990, Esapa et al. 1999, Basolo et al. 2000, Motoi et al. 2000). Another FTC group presents a specific chromosomal translocation t(2;3)(q13;p25), and results in the fusion of two nuclear proteins with transcriptional activity: *PAX8* and peroxisome proliferator-activated receptor γ (*PPAR* γ ; Kroll et al. 2000; Table 1). *PAX8/PPAR* γ rearrangement occurs in ~35% of conventional FTCs, and with a lower prevalence in oncocytic carcinomas also (Dwight et al. 2003, French et al. 2003, Nikiforova et al. 2003).

PDTCs and ATCs can originate from WDTCs and, in particular, from PTCs. Therefore, mutations that occur in the early stages of WDTCs are also observed in PDTCs and ATCs: in PDTCs, *RET* rearrangements (13%) as well as *RAS* (46–55%) and *BRAF* (12–17%) mutations are observed, while in ATCs, only mutations of *RAS* (6–52%) and *BRAF* (25–29%) are reported (Santoro et al. 2002, Garcia-Rostan et al. 2005, Hou et al. 2007, Santarpia et al. 2008; Table 1).

Alterations of *PIK3CA* and *PTEN* (effectors of phosphoinositide 3-kinase) have also been found in thyroid carcinomas. These alterations are generally observed in the later stages of thyroid carcinogenesis and are more frequent in ATCs (16 and 14% respectively) than in WDTCs (2 and 2% respectively) or FTCs (8 and 7% respectively; Paes & Ringel 2008; Table 1). This is in contrast with mutations of the *MAPK* effectors that are preferentially associated with the early stages of thyroid carcinogenesis. More recently, an *AKT1* oncogene mutation in PDTCs has also been observed (Ricarte-Filho et al. 2009). This mutation is commonly associated with *BRAF* mutations and does not overlap with *PIK3CA* mutations in PDTCs (Ricarte-Filho et al. 2009). Impairment of the *p53* tumor suppressor gene function represents a usual feature of ATCs with *p53* mutations being common both in PDTCs (17–38%) and in ATCs (67–88%), but rare or absent (0–9%) in WDTCs (Ito et al. 1992, Donghi et al. 1993, Fagin et al. 1993; Table 1). Moreover, mutations of *CTNNB1* (β -catenin) have been found in 25% of PDTCs and in 66% of ATCs (Garcia-Rostan et al. 2001, Miyake et al. 2001; Table 1).

MicroRNAs (miRNAs or miRs) constitute a class of small endogenous noncoding RNAs of 19–23 nucleotides that negatively regulate gene expression (Bartel 2004). They are an abundant class of gene regulatory molecules in multicellular organisms and modulate the expression of many protein-coding genes (Bartel 2004). They are transcribed as a huge double-stranded primary transcript (pri-miR) by RNA polymerase II. Subsequently, nuclear enzymes Drosha and Pasha convert this precursor into a double-stranded miR precursor of ~70 nucleotides (pre-miR), which is next transported into the cytoplasm by a mechanism involving the protein Exportin. Finally, Dicer enzyme processes this precursor into the 22-nucleotide double-stranded miR. This duplex is then unwinded, and the leading strand, one of the two strands, is incorporated into the RISC. miRs incorporated in the RISC are able to bind to the 3' untranslated region (UTR) of the target mRNAs causing a block of translation or mRNA degradation depending on the level of complementarity (Rhoades et al. 2002, Tang et al. 2003, Ambros 2004, Bartel 2004). Recent studies have clearly demonstrated that they play critical roles in several biologic processes, including differentiation, development, cell growth, and apoptosis, by regulating gene expression through either the inhibition of mRNA translation or the induction of its degradation (Ambros 2004). The analysis of human neoplasias of different tissue origins has shown deregulated miR expression (Calin & Croce 2006, Volinia et al. 2006).

miR expression profile in PTCs

Most studies have focused on the analysis of miR expression profile of PTCs by ‘miRNACHIP’ microarray revealing the upregulation of a set of seven miRs in PTCs compared with the normal thyroid, including *miR-221*, *-222*, *-146*, *-21*, *-155*, *-181a*, and *-181b* (Table 2). Quantitative RT-PCR on a large panel of PTCs confirmed the upregulation of this set of miRs (He *et al.* 2005, Pallante *et al.* 2006, Tetzlaff *et al.* 2007). The upregulation of *miR-221*, *-222*, and *-181b*, observed in all the cases analyzed, showed a value of fold-change that in some cases was higher than 10. Moreover, the upregulation of *miR-221* in unaffected thyroid tissue in several PTC patients suggests miR induction as a possible early event in carcinogenesis (He *et al.* 2005). The critical role of the upregulation of these miRs in thyroid cell transformation was supported by the dysregulation of *miR-221*, *-222*, and *-181b* in the experimental models of thyroid carcinogenesis. Indeed, *miR-221*, *-222*, and *-181b* were not found in the normal thyroid cells PC Cl3, whereas they were highly expressed in the same cells transformed by v-ras-Ha, v-ras-Ki, v-raf, RET/PTC1, RET/PTC3, E1-Abl, E1a-v-raf, middle T of Polyomavirus, and v-mos. These miRs also revealed an elevated expression in the carcinomas of papillary histotype occurring in transgenic animal lines expressing TRK, RET/PTC3 and HPV E7 under the transcriptional control of the thyroglobulin (Tg) promoter, while they

had low expression levels in normal mouse thyroid tissues (Pallante *et al.* 2006).

Since several genetic lesions have been detected in PTCs, the miR expression profile was analyzed in PTCs already selected for genetic mutations. This study revealed a strong correlation between the miR expression and the mutational status. *MIR-187* was expressed at high levels in PTCs harboring *RET/PTC* rearrangements, whereas *miR-221* and *-222* were found at the highest level in *BRAF*- and *RAS*-positive PTCs and those with no known mutations. *RAS*-positive PTCs expressed the highest amount of *miR-146*. Essentially, it can be stated that *BRAF*- and *RET/PTC*-positive tumors segregated into separate clusters, while *RAS*-positive tumors did not form separate clusters (Nikiforova *et al.* 2008). However, PTCs keep some changes in the expression profile independently from the mutational status. The mechanism by which these miRs are upregulated in PTCs is still under investigation. In all the papers reported so far, no gene amplification or changes in the methylation status have been found, making us assume that other epigenetic mechanisms may be responsible for miR overexpression (Pallante *et al.* 2006).

It is also of note that even though PTCs include different subgroups such as classical PTCs, Hurthle cell variant of PTCs, and tall cell, follicular variant of PTCs, a clear correlation between miR profile and histological subtypes has not been evidenced so far.

Table 2 miRs aberrantly expressed in human thyroid carcinomas of follicular cell origin

Tumor histotype	miR deregulation	Up/down	References
PTC	146, 221, 222, 21, 220, 181a, 155	Up	He <i>et al.</i> (2005)
	26a-1, 345, 138, 219	Down	He <i>et al.</i> (2005)
	222, 221, 181b, 220, 213	Up	Pallante <i>et al.</i> (2006)
	187, 221, 222, 146b, 155, 122a, 31, 205, 224	Up	Nikiforova <i>et al.</i> (2008)
	1, 191, 486, 451	Down	Leone <i>et al.</i> ^a
	146b, 221, 222	Up	Chen <i>et al.</i> (2008)
	221, 222, 21, 31, 172, 34a, 213, 181b, 223, 224	Up	Tetzlaff <i>et al.</i> (2007)
	218, 300, 292, 345, 30c	Down	Tetzlaff <i>et al.</i> (2007)
	339, 224, 205, 210, 190, 328, 342	Up	Nikiforova <i>et al.</i> (2008)
	31, 339, 183, 221, 224, 203	Up	Nikiforova <i>et al.</i> (2008)
FTA Conventional type	339, 224, 205, 210, 190, 328, 342	Up	Nikiforova <i>et al.</i> (2008)
FTA Oncocytic type	31, 339, 183, 221, 224, 203	Up	Nikiforova <i>et al.</i> (2008)
FTC Conventional type	187, 224, 155, 222, 221, 146b	Up	Nikiforova <i>et al.</i> (2008)
FTC Oncocytic type	187, 221, 339, 183, 222, 197	Up	Nikiforova <i>et al.</i> (2008)
	192, 197, 328, 346	Up	Weber <i>et al.</i> (2006)
PDTC	187, 221, 129, 222, 146b, 339, 183	Up	Nikiforova <i>et al.</i> (2008)
ATC	30d, 125b, 26a, 30a-5p	Down	Visone <i>et al.</i> (2007b)
	302c, 205, 137, 187, 214, 155, 224, 222, 221	Up	Nikiforova <i>et al.</i> (2008)
	21, 146b, 221, 222	Up	Mitomo <i>et al.</i> (2008)
	26a, 138, 219, 345	Down	Mitomo <i>et al.</i> (2008)
	17-5p, 17-3p, 18a, 19a, 20a, 19b, 92-1, 106a, 106b	Up	Takakura <i>et al.</i> (2008)

^aLeone V, D'Angelo D & Fusco A, unpublished observation.

The diagnostic utility of miR expression was explored in order to translate the analysis of these molecules in clinical applications by analyzing the expression of the miRs overexpressed in fine-needle aspiration (FNA) specimens of PTCs. Pallante et al. (2006) found *miR-221*, *-222*, and *-181b* overexpression in seven of eight lesions corresponding to thyroid nodules, which were diagnosed as PTC after surgery. Chen et al. (2008) also demonstrated the possible use of *miR-146b* as a marker for diagnosing PTCs in both FNA and surgical specimens. In another study, the expression of a set of seven miRs (*miR-187*, *-221*, *-222*, *-224*, *-146b*, *-155*, and *-197*) was analyzed in FNA specimens of thyroid carcinomas and of hyperplastic nodules (Nikiforova et al. 2008). The evaluation of these miRs was very useful in differentiating thyroid cancer from hyperplastic nodules. In fact, the supervised prediction analysis by linear discriminant analysis failed to distinguish only one of 41 malignant tumors and hyperplastic nodules (Nikiforova et al. 2008). These results indicate the evaluation of these miRs as a potential application for the diagnosis of human thyroid neoplasias.

Role of the *miR-221/222* cluster in thyroid carcinogenesis

MiR-221 and *-222* are the most consistently upregulated miRs in PTCs. They are very similar in sequence, clustered on chromosome X, and are likely transcribed as polycistrons (Ciafrè et al. 2005). Functional studies were performed to understand their role in thyroid carcinogenesis. Using a colony assay, it was demonstrated that the *miR-221* and *-222* overexpression on thyroid carcinoma cell lines increased the number of colonies (>2-fold) in comparison to the same cells transfected with an empty vector. Opposite results were obtained when thyroid carcinoma cell lines were treated with the *miR-221* and *-222* antisense oligonucleotides (Pallante et al. 2006). These results suggest that *miR-221* and *-222* overexpression plays a significant role in thyroid carcinoma cell proliferation.

Several targets for the *miR-221/222* cluster have been identified. One of these is *c-KIT* (Felli et al. 2005), also called *CD117*, a cytokine receptor for the stem cell factor expressed on the surface of hematopoietic stem cells as well as in other cell types. In most PTCs, the loss of *c-KIT* transcript and c-KIT protein has been described (Natali et al. 1995); however, its role in thyroid carcinogenesis is still unknown.

More recently, the *CDKN1B* (*p27^{Kip1}*) gene was identified as a target of *miR-221* and *-222* (Galardi et al. 2007, Visone et al. 2007a). *p27^{Kip1}*, member of

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). Cip/Kip family together with INK4 proteins (*p16^{INK4a}*, *p15^{INK4b}*, *p18^{INK4c}*, and *p19^{INK4d}*) makes part of the cyclin-dependent kinase (CDK) inhibitors (CDKI; Serrano et al. 1993, Guan et al. 1994, Hannon et al. 1994, Hirai et al. 1995). These proteins contrast the activities of CDKs which are involved in the regulation of the mitogen-dependent progression through gap phase 1 (G1 phase) and initiation of DNA synthesis (S phase) during the mammalian cell division cycle (Kaldis 2007). Reduced *p27^{Kip1}* protein levels were found in a high number of human thyroid malignancies in the absence of significant changes in *p27* mRNA levels (Baldassarre et al. 1999). When transfected into PTC cell line, *miR-221* and/or *-222* decreased *p27^{Kip1}* protein levels, which were increased by the treatment with their inhibitors. Interestingly, no significant changes in the *p27^{Kip1}* mRNA levels were found in the cells transfected with either *miR-221* and *-222* or their inhibitors, excluding their role in *p27^{Kip1}* mRNA degradation. The regulation of *p27* by *miR-221* and *-222* was direct, as they negatively regulated the expression of the 3' UTR-based reporter construct from the *p27^{Kip1}* gene, and was dependent on two target sites in this mRNA region (Visone et al. 2007a). Consistently with the key role of *p27^{Kip1}* in the cell cycle, particularly in the cell growth arrest at the G1/S transition, the enforced expression of *miR-221* and *-222* stimulates thyroid carcinoma cells to overcome the G1/S block. Therefore, the role of the *miR-221/222* cluster overexpression may account for the reduced *p27^{Kip1}* expression in several tumors. Consistently, a significant inverse correlation between *p27^{Kip1}* protein levels and *miR-221* and *-222* expression was found in PTCs (Visone et al. 2007a). Other studies have shown that *miR-221* can also target *CDKN1C/p57* that has a critical role in the cell-cycle control (Fornari et al. 2008). Therefore, the upregulation of *miR-221/222* significantly modifies the cell cycle of thyroid cells, and when associated with a genetic lesion involving the MAPK pathway, could lead to the malignant phenotype.

MiR-221 and *-222* are also able to impair tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL)-dependent apoptosis by inhibiting the expression of key functional proteins (Garofalo et al. 2008). Recent results indicate that *miR-222* plays an important role in cancer cell invasion. Indeed, ectopic transfection of *miR-222* reduced the expression of matrix metalloproteinase 1 (*MMPI*) and manganese superoxide dismutase 2 (*SOD2*) in oral tongue

squamous cell carcinoma cell line. Interestingly, *SOD2* knockdown by siRNA led to the downregulation of *MMP1* expression. Consequently, *miR-222* would regulate the *MMP1* expression through both direct *cis*-regulatory mechanism (targeting *MMP1* mRNA) and indirect *trans*-regulatory mechanism (indirect controlling of *MMP1* gene expression by targeting *SOD2*; Liu *et al.* 2009).

In conclusion, the overexpression of *miR-221* and *-222* targets critical genes, making their overexpression a crucial factor in the process of thyroid oncogenesis.

***miR-146* in thyroid cancer**

miR-146 represents one of the most upregulated miRs in PTCs. It identifies two different miRs that differ only for two nucleotides: *miR-146a* (on chromosome 5q33) and *-146b* (on chromosome 10q24). Additional forms of mature miRs can originate from the passenger strand of *miR-146b* (Jazdzewski *et al.* 2009).

Both *miR-146* isoforms are overexpressed in PTC samples, with *miR-146b* showing a higher expression than *miR-146a* in several specimens (He *et al.* 2005, Jazdzewski *et al.* 2008). These two miRs are differently regulated: lipopolysaccharide is able to strongly induce *miR-146a*, but not *miR-146b*, and the promyelocytic leukemia zinc finger protein transcription factor is able to downregulate only *miR-146a* (Labbaye *et al.* 2008). Moreover, recent data demonstrate that the control of *miR-146a* and *-146b* expression differs depending on cell type and environment (Hurst *et al.* 2009).

It has been reported that *miR-146a* and *-146b* can interact with a domain in the exon 18 region of the *c-KIT* mRNA. Intriguingly, there is a single-nucleotide polymorphism (SNP; 2607G>C) located in the crucial region of the *c-KIT* mRNA pairing with *miR-146*. This event could lead to changes in the duplex conformation between miR and mRNA and result in hybridization with a different region, modulating the expression of the target gene in a different manner (He *et al.* 2005).

Since *miR-146a* and *-146b* show high sequence homology, they share many predicted targets, but each of them has specific targets. *miR-146* suppresses nuclear factor- κ B (*NF- κ B*) activity that regulates its expression (Taganov *et al.* 2006, Bhaumik *et al.* 2008). Moreover, the breast cancer metastasis suppressor 1 upregulates *miR-146a* and *-146b*, which suppress breast cancer metastasis since they are able to inhibit migration and invasion likely reducing the signaling through the *NF- κ B* pathway (Hurst *et al.* 2009).

This appears to be a rather interesting novel principle of local feedback regulatory circuits.

Because the role of *NF- κ B* in thyroid carcinogenesis has been clearly well established already (Visconti *et al.* 1997, Pacifico *et al.* 2004, McCall *et al.* 2007), we can assume that the *miR-146* overexpression might contribute to the development of PTCs.

Other targets of *miR-146* are interleukin-1 (IL-1) receptor-associated kinase 1 (*IRAK1*; Taganov *et al.* 2006, Bhaumik *et al.* 2008), TNF receptor-associated factor 6 (*TRAF6*; Taganov *et al.* 2006, Bhaumik *et al.* 2008), *IL-8*, (Bhaumik *et al.* 2008), *IL-6*, (Bhaumik *et al.* 2008), C-X-C chemokine receptor type 4 (*CXCR4*; Labbaye *et al.* 2008), *MMP9* (Bhaumik *et al.* 2008), and epidermal growth factor receptor (Griffiths-Jones *et al.* 2008, Hurst *et al.* 2009). Recently, a role for *miR-146* has been proposed in the control of toll-like receptor and cytokine signaling through a negative feedback regulation loop involving downregulation of *IRAK1* and *TRAF6* protein levels (Taganov *et al.* 2006, 2007). In the *pre-miR-146a*, there is a common G/C polymorphism designated rs2910164 (Jazdzewski *et al.* 2008). Because of this SNP in its sequence, *miR-146a* gene generates two transcript isoforms: *miR-146a-G* and *miR-146a-C*. Therefore, while two mature miRs originate from GG and CC homozygotes (*miR-146a* and *miR-146a-G* or *146a-C* respectively from the passenger strand), three mature miRs arise from GC heterozygotes (*miR-146a* and both *miR-146a-G* and *-146a-C*; Jazdzewski *et al.* 2009). The G/C polymorphism also affects the final production of mature *miR-146a* by reducing the stability of the pri-miR, decreasing the efficiency of processing the pri-miR into pre-miR, or the pre-miR into mature miR (Jazdzewski *et al.* 2008). The G/C polymorphism shows a marked difference in genotype distribution and seems to affect the predisposition to PTCs, and in fact, an increased risk to develop PTCs is associated with the GC heterozygous status (Jazdzewski *et al.* 2008). Moreover, 4.7% of analyzed tumors showed somatic mutations of the SNP from both homozygous states to heterozygosity (Jazdzewski *et al.* 2008). This is a rare phenomenon (overdominance) in which heterozygosity is a genetic risk rather than homozygosity (Jazdzewski *et al.* 2008, 2009).

Since the G/C polymorphism is located in the region of complementarity between the *miR-146a* and the target mRNAs, different mechanisms might be envisaged to explain the predisposition to PTCs by the GC state of the SNP:

- a) The different isoforms of *miR-146a* could have different targets or act with different efficiency on them. Indeed, *miR-146a-G* reduces the expression of *IRAK1* and *TRAF6* more efficiently than *miR-146a-C* (Jazdzewski et al. 2008).
- b) In the heterozygous state, there are three sets of miRs and therefore a higher number of target genes.

miRs downregulated in PTCs

Our group, by analyzing more PTC samples, has recently found other miRs deregulated in PTCs (Table 2), among which are *miR-1*, *-191*, *-486*, and *-451*. These miRs showed lower expression levels in most of the PTCs analyzed compared to normal thyroid tissue. We have found that *miR-1* is able to target *CXCR4* (V Leone, submitted for publication). This protein, also called fusin, is an α -chemokine receptor specific for stromal cell-derived factor-1 (also called *CXCL12*), a molecule endowed with potent chemotactic activity for lymphocytes (Bleul et al. 1996). *CXCR4* is frequently overexpressed in PTCs and plays a major role in the mechanism of lymph node metastasis from primary tumor (Castellone et al. 2004). Therefore, the downregulation of *miR-1* may have a critical role in the tropism of PTC cells to the local lymph nodes. The tumor suppressor role of *miR-1* in thyroid carcinogenesis is further supported by its ability to reverse the tumorigenic properties (such as growth, motility, migration, clonogenic survival, and tumor formation in athymic mice) of lung cancer cells (Nasser et al. 2008).

miR expression in FTCs

Even though most of the studies conducted so far on miR expression in thyroid cancer have focused on PTCs, recently several papers have reported miR deregulation in FTCs also. It has been shown by Nikiforova et al. (2008) that a different signature is associated with follicular adenoma and carcinoma both in conventional variants and in oncocytic ones. The most highly upregulated miRs in conventional FTCs were *miR-187*, *-224*, *-155*, *-222*, and *-221*, and those in oncocytic variants were *miR-187*, *-221*, *-339*, *-183*, *-222*, and *-197*, whereas the most highly upregulated miRs in conventional FTAs were *miR-339*, *-224*, *-205*, *-210*, *-190*, *-328*, and *-342*, and those in oncocytic variants were *miR-31*, *-339*, *-183*, *-221*, *-224* and *-203* (Nikiforova et al. 2008). Interestingly, the upregulation of these miRs was not observed in hyperplastic

nodules. In another study, Weber et al. (2006) identified four miRs differentially expressed between FTCs and FTAs (*miR-192*, *-197*, *-328*, and *-346*). Moreover, *in vitro* functional analyses showed that the overexpression of *miR-197* and *-346* induced marked cell proliferation in HEK293T cells, while their inhibition caused growth arrest in human thyroid carcinoma follicular cells (FTC133; Weber et al. 2006). They also confirmed the regulation of three target genes by *miR-197* (*ACVR1* and *TSPAN3*) and *-346* (*EFEMP2*). *EFEMP2* seems to have tumor suppressor functions (Gallagher et al. 2001, Argraves et al. 2003), whereas *ACVR1* is involved in the control of cell growth (Schulte et al. 2001). *TSPAN3* belongs to the tetraspan superfamily, whose members have been shown to be inversely correlated with the acquisition of metastatic potential in melanoma (Schulte et al. 2000, Boucheix et al. 2001).

miR expression in anaplastic thyroid carcinomas

As far as ATCs are concerned, a significant downregulation of *miR-30d*, *-125b*, *-26a*, and *-30a-5p* has been described (Table 2; Visone et al. 2007b). The possible role of these miRs in the generation of ATCs seems to be validated by the mouse models of carcinogenesis. Indeed, they were downregulated in ATCs developing in transgenic animal mice expressing SV40 *large T* oncogene under the transcriptional control of the the Tg promoter (Ledent et al. 1991). A role of *miR-30* and *-125b* downregulation in thyroid cancer progression seems to be supported by recent results showing a decreased expression of these miRs in other malignant neoplasias, such as prostate cancer for both the miRs (Ozen et al. 2008) and oral squamous cell carcinoma for *miR-125b* (Henson et al. 2009). Recent results report a role of *miR-30a* in regulating Beclin 1, the mammalian homologue of yeast *Atg6*, a key autophagy-promoting gene that plays a critical role in the regulation of cell death and survival of various cell types. Consistently, treatment of tumor cells with *miR-30a* results in the decreased autophagic activity (Zhu et al. 2009). Among the potential targets of *miR-125b*, there is the sel-1 suppressor of lin-12-like (*SEL1L*) protein, upregulated in prostate carcinomas, which may play a role in breast and pancreatic cancer aggressiveness (Biunno et al. 2006). Just recently, the tumor suppressor role of the *miR-26a* also appears to be validated by the finding of a reduced expression of this miR in human liver carcinomas in comparison to normal liver cells. Moreover, it has been demonstrated that the enforced expression of *miR-26a* in

liver cancer cells induces cell-cycle arrest associated with direct targeting of *cyclins D2* and *E2*, and systemic administration of this miR in a mouse model of human hepatic carcinoma, using adeno-associated virus, results in the inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression (Kota *et al.* 2009). More recently, Schwertheim *et al.* (2009) confirmed the downregulation of these miRs and *let-7c* in a different panel of ATC samples.

A recent study revealed an upregulation of several miRs in ATCs (Table 2). Interestingly, these miRs were found to be overexpressed also in WDTCs deriving from follicular cells, and some of them such as *miR-302c*, *-205*, and *-137* resulted overexpressed in comparison to hyperplastic nodules (Nikiforova *et al.* 2008).

Another study (Mitomo *et al.* 2008) revealed that *miR-21*, *-146b*, *-221*, and *-222* were overexpressed in ATCs (Table 2). Among the downregulated miRs were found *miR-26a*, *-138*, *-219*, and *-345*. A potential target of *miR-138* is the human telomerase reverse transcriptase (*hTERT*) whose overexpression has been associated with dedifferentiation, tumor stage, and increased metastatic and invasive phenotypes (Ito *et al.* 2005, Mitomo *et al.* 2008). These results suggest that loss of *miR-138* expression may partially contribute to the gain of hTERT protein expression in ATC cells (Mitomo *et al.* 2008).

It has been recently reported by Takakura *et al.* (2008) that the *miR-17-92* cluster of seven miRs (*miR-17-5p*, *-17-3p*, *-18a*, *-19a*, *-20a*, *-19b*, and *-92-1*) as well as *miR-106a* and *-106b* was overexpressed in ATC cell lines. *miR-17-3p* and *-17-5p* resulted overexpressed also in human ATC samples compared to normal tissue. The inhibition of *miR-17-3p*, *-17-5p*, and *-19a* resulted in a reduced cell growth, thus suggesting an oncogenic potential of these miRs. In this study, retinoblastoma protein (*RB1*) and *PTEN*, predicted to be putative targets in previous studies (Griffiths-Jones *et al.* 2006), are now confirmed to be targets of *miR-17-5p* and *-19a* (Takakura *et al.* 2008).

MiR-17 family of miR has been demonstrated to modulate fibroblast growth factor 10 (*FGF10*)–fibroblast growth factor receptor 2b (*FGFR2b*) downstream signaling by specifically targeting the signal transducer and activator of transcription 3 (*STAT3*) and the *MAPK14*, hence regulating E-cadherin expression, which in turn modulates epithelial bud morphogenesis in response to *FGF10* signaling

(Carraro *et al.* 2009). Therefore, the overexpression of *miR-17* family members might contribute to the development of a significant number of ATC cases.

Conclusions and perspectives

Several studies have shown deregulation of miR expression in human thyroid carcinomas, and functional studies support a critical role of miRs in thyroid carcinoma development.

The generation of transgenic mice overexpressing these miRs under the control of a thyroid-specific promoter (i.e. Tg), and also their crossing with transgenic mice expressing the most frequently activated oncogenes (*RET/PTC3*, *TRK-T1*, and *N-RAS*) in thyroid carcinomas, could help clarify their role in thyroid carcinogenesis. The deregulated expression of these miRs together with their biological role suggests a correlation with diagnosis, prognosis, and therapeutic approaches. However, even though two studies show that the evaluation of the expression of a set of miRs can allow the distinction between thyroid cancer and hyperplastic nodules, a miR signature that could clearly distinguish benign FTAs from FTCs – the major challenge in the preoperative diagnosis – is not available at the moment.

Hopefully, taking advantage of the miRs identified recently, further studies may be helpful in achieving this important aim.

Inhibition of miRs such as *miR-221*, *-222*, and *-146*, with ‘antagomirs’ or ‘locked nucleic acid-modified anti-miRs’ (LNA-antimiRs; to be tested in animal models of thyroid carcinogenesis), is an attractive direction for therapy. Restoration of downregulated miRs as well could be a tool to improve patient care and response to treatment. Just recently, Kota *et al.* (2009) have reported that restoration of the *miR-26* (a miR constantly downregulated in ATCs) expression in hepatocellular carcinoma cells using an adeno-associated virus results in the inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and remarkable protection from disease progression. This result suggests a possible use of *miR-26a* restoration in ATC that is well known to be refractory to any conventional radiotherapy and chemotherapy. Moreover, the possibility to antagonize the liver-expressed *miR-122* in nonhuman primates by the simple systemic delivery of LNA-antimiRs (Elmén *et al.* 2008) makes the utilization of systemically delivered LNA-antimiRs for the therapy of thyroid and nonthyroid carcinomas not futuristic.

Declaration of interest

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

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