Review

The Role of MicroRNAs in Thyroid Carcinomas

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Abstract. *Thyroid cancers (TCs) are the most common malignancies of endocrine organs. They originate from cells of different origin within the thyroid gland, which is located at the base of the neck. Several forms of TCs have been classified and great variability is observed in molecular, cellular and clinical features. The most common forms have favorable prognosis but a number of very aggressive TCs, which are characterized by a less differentiated cellular phenotype, have no effective treatment at the moment. While TC causes are not completely understood, many genetic factors involved in their onset have been discovered. In particular, activating mutations of BRAF, RET or RAS genes are known to be specifically associated with TC initiation, progression and outcome. The involvement of microRNAs in thyroid neoplasms has recently changed the paradigm for biomarker discovery in TC, suggesting that these small non-coding RNAs could be used to develop, refine or strengthen strategies for diagnosis and management of TCs. In this review, the importance of microRNA profiling in TC is explored suggesting that these molecules can be included in procedures that can perform better than any known clinical index in the identification of adverse outcomes.*

Thyroid cancer (TC) is a group of uncommon neoplastic diseases affecting the thyroid gland. Among carcinomas of follicular cell origin, three categories of TCs can be defined

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according to specific histological features: well-differentiated thyroid carcinomas (WDTCs), poorly differentiated thyroid carcinomas (PDTCs) and undifferentiated thyroid carcinomas (UDTCs) (1). A sequential model in which UDTCs represent the final step of WDTC progression through the spectrum of PDTC has been suggested by evidence of pre- or co-existing WDTCs with less differentiated types that also share the original genetic alteration (2).

Medullary thyroid cancer (MTC), unlike the previously mentioned, is a neuroendocrine tumor that arises from parafollicular C cell and constitutes types fewer than 5% of all TC cases (3).

WDTCs are the most common TC forms and include papillary thyroid carcinoma (PTC), which accounts for about 75-80% of worldwide TC occurrences, and the less frequent (10-15%) follicular thyroid carcinoma (FTC). PTC differential diagnosis from FTC is determined by histology. PTC diagnosis relies on the presence of follicular cell differentiation, typically with papillary and follicular structures, as well as characteristics nuclear changes, such as large size, pale staining, 'ground glass' appearance, irregular outlines or inconspicuous nucleoli. FTC is characterized by evidence of follicular differentiation that lacks papillary architecture and typical nuclear features of PTC (4, 5). Even if WDTCs are usually considered an homogeneous group of neoplastic diseases in terms of outcome, follicular cancer is often more aggressive, leading less favorable survival rates. On the other hand, much evidence suggests that tumor histology cannot be considered alone as a determinant of WDTC prognosis and patient- (*e.g.* sex and mainly age) and tumor- (*e.g.* TNM or size) related factors are usually taken into account to define risk categories (6-9).

PDTCs are more aggressive than WDTCs and comprise nonfollicular, nonPTCs. While PDTCs can be easily placed between differentiated and undifferentiated carcinomas in term of prognosis, a rigorous and widely accepted histologic

Table I. *Prevalence, clinicopathological features and most frequent genetic defects of thyroid neoplasms. Genetic alterations are in order of prevalence from the most to the less frequent.*

Tumor type	Prevalence	Lymph node metastasis	Distant metastasis	5-Year survival	Genetic alteration	References
PTC	75-80%	<50%	5-7%	~ 100% stage I -II 93% stage III 51% stage IV	*BRAF* mutation *RET* rearrangement *NTRK1* rearrangement *RAS* mutation *TP53* mutation (uncommon) *BRAF* rearrangement (uncommon)	(28, 30, 77, 97, 98, 99, 101, 102)
FTC	10-15%	<5%	20%	~ 100% stage I -II 71% stage III 50% stage IV	*RAS* mutation *PPARγ* rearrangement *TP53* mutation (uncommon)	(44, 101, 102)
PDTC	<7%	30-80%	30-80%	50%	*TP53* mutation *RAS* mutation β-Catenin mutation *RET* rearrangement *BRAF* mutation	(28, 30, 98, 99, 101)
UDTC	2%	40%	20-50%	7%	*TP53* mutation β-Catenin mutation *RAS* mutation *BRAF* mutation	(28, 30, 46, 101)
MTC	<5%	50%	15%	86%	*RET* mutation	(100)

PTC: Papillary thyroid carcinoma, FTC: follicular thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; UDTC: undifferentiated thyroid carcinoma; MTC: medullary thyroid carcinoma.

classification is still controversial. According to some authors, they can be classified on the base of two major characteristics: presence of a solid-trabecular-insular (STI) growth pattern and absence of conventional nuclear features that define papillary carcinomas (10, 11). Some authors, on the other hand, propose histological grading as the only determinant irrespective of growth pattern (12). Controversial designations also arise from the different thresholds used by authors in defining classification requirements. Many authors provide evidence of the impact of different cutoff usage for the identification of STI areas in comparing prognoses (13). For this reason, PDTCs can be considered quite rare and heterogeneous tumors and their prevalence can only be roughly estimated at between 1.5% and 6% of malignant TCs (10, 14).

Anaplastic thyroid carcinoma (ATC) is a highly aggressive form of UDTC. ATCs constitute the most uncommon subgroup of TCs and are characterized by rapid growth, metastatic invasion and, consequently, a dramatically unfavorable prognosis. The 5-year relative survival rate for anaplastic carcinoma has been defined at round 7%. All the other forms of TC have a much better prognosis, with 5-year relative survival rates of 50% for PDTC (15, 1), 86% for MTC (16) and from 50% to near 100%, according to stage, for WDTCs (17).

Many diagnostic algorithms that evaluate histological, surgical and molecular features of the thyroid neoplasms can often be used to easily define TC subgroups and patient risk. Nevertheless, in some cases, diagnosed TC tumors show intermediate or confounding profiles that make the classification more difficult. Some of the above- mentioned disease groups present intermediate characteristics and comprise tumors that are heterogeneous for prognosis or sensitivity to treatment. Many efforts have been made to improve the accuracy of disease profiling for a better classification of tumors that may actually behave differently, despite their apparent histological similarities; however, TC management is still an important medical challenge. The identification of unambiguous biological and molecular features that can be used to discriminate indolent from aggressive disease, for example, would be fundamental in defining clinically useful predictor risks. On this basis, it is interesting to note that deregulation of microRNA (miRNA) function was recently identified as an important driver for tumor development and progression in TCs (18, 20). miRNAs are a class of small noncoding RNAs that are able to regulate gene expression through mRNA cleavage or translational repression. The specificity of miRNA–mRNA interaction is directed by nucleotide sequence complementarity between the miRNA seed region and its corresponding binding sequence on the target mRNA. The flexibility of this recognition system allows miRNA to target multiple molecules and mRNAs to be regulated simultaneously by more than one miRNA, therefore

constituting the basis for a complex and diffused regulatory network. These molecules finely regulate many biological processes (including proliferation, apoptosis and differentiation) and deeply influence pathological pathways (20).

Molecular Biology and Biomarkers of TCs

The growth of follicular thyroid cells is physiologically regulated by the thyroid-stimulating hormone (TSH or thyrotropin). TSH receptor, upon thyrotropin binding, activates the GSα-adenylyl cyclase-cyclic AMP (cAMP) cascade, triggering cell differentiation and proliferation through transcriptional activation of specific molecular effectors. Even though mutations that constitutively activate TSH receptor or GSα have been identified in 60-70% of benign adenomas (21-23), the hypothesis of a causative role in malignant transformations of thyroid cells has been rejected (24-27). While the constitutive activation of cAMP-elicited transduction cascade alone is not sufficient for neoplastic transformation, many genetic abnormalities have been described in different histological types of TC (Table I).

In WDTCs, the vast majority of cases present genetic alterations affecting the MAPK signaling pathway. In particular, activating mutations of *BRAF*, *RET* or *NTRK1* genes have been identified as causative and mutually exclusive events in PTC onset. The T1799A transversion mutation of *BRAF* that produces the V600E variant of the encoded kinase occurs in about 45% of sporadic PTCs (28). It has been demonstrated that this mutation is associated with a poorer outcome and it can be used as a predictor of clinical features, such as extranodal invasion, lymph node metastasis, advanced staging and disease recurrence (29). While *BRAF* mutations have not been found in FTC, they are present in 13% of PDTC. They have been also observed more frequently in UDTC with a PTC component than in those without such a component (30). The expression of hexogen V600E *Braf* in mouse thyroid cells produces PTC with some of the PDTC specific features (31). While the V600E variant of *BRAF* is the most common alteration in sporadic PTC, other variants (*e.g.* the K601E for the follicular variant papillary carcinomas and the in-frame VK600-1E deletion for the solid variant papillary carcinoma) define a phenotype specific range of genetic alterations targeting *BRAF* exons (32). Much evidences (33-35) suggests that deregulated activation of the MAPK cascade can increase genomic instability of TC cells, thus promoting the acquisition of additional somatic mutations during TC progression.

The receptor-tyrosine kinase encoded by the *RET* gene is an essential molecular actor required for renal organogenesis and enteric neurogenesis. It has been shown that while gain-of-function *RET* mutations are associated with MTC (36), the chromosomal rearrangements that originate a family of chimeric RET oncoproteins are involved in PTC development. At least 10 chimeric PTC- associated *RET* variants have been identified in PTC (37) and all of them result from the fusion of the 3'- terminal sequence of *RET* that encodes for a tyrosine kinase domain with the 5' portion of different genes.

The neurotropic receptor-tyrosine kinase (*NTRK1*) gene encodes for a tyrosine-kinase receptor that is activated by NDF. Its expression is typically neural and usually restricted to sympathetic, trigeminal and dorsal root ganglia or to cholinergic neurons of the basal forebrain and the striatum (38). This receptor, upon NGF binding, transduces the signal activating several pathways, including ERK, PI3K and the PLCγ cascades. *NTRK1* rearrangements observed in TC, like those in *RET-PTC*, involve the tyrosine kinase domain of NTRK1 that is fused to another gene. Only three fusion partners, which have practically identical prevalence in sporadic PTC, have been identified so far: *TFG*, *TPM3* and *TPR*.

RAS mutations are the second most abundant mutations identified in TCs. They comprise mutations affecting a family of three homologous isoforms *HRAS*, *KRAS* and *NRAS*, the latter of which is the most frequently mutated in TC. These membrane-associated guanine nucleotide-binding proteins transduce and amplify extracellular signals to initiate important cellular responses. The type of guanine nucleotide bound to RAS regulates their activation state: while GTP promotes RAS activity, GDP inhibits its function. The GTP-ase function of these proteins promotes their deactivation in a typical feedback manner: when GDP is bound to RAS, the resulting conformation is inactive. The physiological role of *RAS* genes is dual: they are involved in the activation of both MAPK and PI3K-AKT pathways. TC-related *RAS* variants, which predominantly exhibit mutations of codons 12, 13 and 61, preferentially activate the PI3K-AKT axis as suggested by the observed association between the occurrence of these mutations and phospho-AKT increase (39). Oncogenic variants produced by point mutation in codons 12 and 13 are characterized by increased affinity for GTP, while those resulting from mutations of codon 61 show deficiency in the autocatalytic GTP-ase function (40). Among WDTCs, *RAS* mutations have been observed more frequently in tumors with follicular histology (FTC and follicular variant of PTC) than in typical papillary TCs (41, 42). In FTC, *NRAS* codon 61 mutations are also associated with distant metastasis, supporting the hypothesis that aberrantly active proteins participate in providing a more aggressive clinical phenotype. *RAS* mutations have been observed also in PDTCs and UDTCs with variable frequencies (1).

Some authors suggested that *RAS* mutations and rearrangements involving the peroxisome proliferator-activated receptor-γ (*PPARγ*) and paired box 8 (*PAX8*) genes, are, in FTC, mutually exclusive events with distinct molecular mechanisms (43, 44). It has been shown that the *PPARγ* gene, which encodes for a steroid nuclear hormone receptor that forms heterodimers with retinoid X receptor,

can generate a chimeric fusion gene with *PAX8*, a transcription factor normally involved in thyroid follicular cell development. The *PPARγ–PAX8* chimera acts as a negative dominant affecting the transcriptional activity of the wild type *PPARγ* gene. The fusion oncoprotein contributes to malignant transformation in FTC and some authors indicate that it promotes the vasculo-invasive phenotype.

Cadherin-associated protein beta 1, encoded by the gene *CTNNB1* and usually called β-catenin, is a protein that by acts -regulating both cell-cell adhesion and signal transduction through the Wnt pathway. This transduction cascade is essential for embryogenesis and integrates signals from other molecular pathways (retinoic acid, fibroblast growth factor, transforming growth factor beta 1, bone morphogenetic protein) within different cell types and tissues. The cascade starts when the Wnt ligand binds to the extracellular portion of the Frizzled receptor that in turn activates a number of molecular partners releasing β-catenin from its post translational regulation exerted by proteasomal degradation. The subsequent accumulation of cytosolic β-catenin allows the protein to translocate to the nucleus where it promotes the transcription of target genes. Aberrant expression of β-catenin has been reported in many tumors (45). Increased cytoplasmic β-catenin is observed in TC but its increased nuclear localization, which is promoted by specific mutations, appears to be limited to PDTC and UDTC (46). Different authors recently reported a clear functional link between RET oncoprotein activation and the modulation of downstream β-catenin axis (47, 48).

The overall picture of molecular events involved in TC initiation and progression has to be completed adding the fundamental details represented by the regulatory layer controlled by miRNAs.

In the recent years, these small endogenous regulators have been clearly identified as key molecular components in cancer and tumor miRNA profiles have been shown to be capable of defining efficiently relevant pathologic subtypes, patient response and survival.

miRNA as Biomarkers

More than 2,000 miRNAs have been identified in humans (49). It has been shown that about 50% of human miRNA genes are located in cancer-associated genomic regions or in fragile sites (50), supporting their contribution as key drivers of neoplastic transformation that follows genetic injuries. Mutations in miRNA target sites that lead to an incorrect mRNA recognition may induce severe phenotypic consequences and may promote cancer initiation. (51, 52). Chromatin remodeling may also affect miRNA expression through the methylation of promoter sequences. (53).

The first evidence of the role of miRNAs in cancer was reported for chronic lymphocytic leukemia (54), where the cluster containing miR-15 and miR-16 is frequently deleted or down-regulated in cancer cells. During the past decade, many studies reported that miRNAs are often differentially modulated in cancer cells, defining severe miRNA expression patterns that were specifically associated with histological variants and tumor prognosis (55-58). Moreover, it has been accepted that miRNAs can be directly controlled by oncogenic transcription factors (59).

A common feature of tumor-related miRNA profiles is the frequent down-regulation of miRNAs in cancer cells, while their over expression is a much less frequently observed phenomenon. In physiological conditions, indeed, miRNA expression increases, as during cell differentiation. Lower expression levels in cancer cells may be coherent with the acquisition of a less differentiated state, a common feature in malignant transformation. The transcriptional inhibition of miRNA genes in cancer cells can be exerted through repression by oncogenic transcription factors (60), or through the alteration of the miRNA biogenesis pathway (61, 62).

Some authors highlighted the importance of miRNA biogenesis regulation in TC (63). miRNAs are transcribed mainly by RNA polymerase II or more rarely by RNA polymerase III as double-stranded precursors called primary miRNA (pri-miRNA). These molecules, which are up to several thousands nucleotides long, are characterized by distinctive secondary stem-loop structure. Inside the nucleus, pri-miRNAs are enzymatically cleaved into smaller (70- to 80-nucleotide-long) molecules, defined pre-miRNAs, that present hairpin structure due to reverse complementary regions in their sequence. A multicomponent complex called a microprocessor, whose core components are Drosha and DGCR8 enzymes, carries out this cleavage. (64). DGCR8 (also known as PASHA in invertebrates) stabilizes the pri-miRNA, while Drosha, an RNAse type III protein, cuts it into the pre-miRNA. The pre-miRNAs are then transported to the cytoplasm by their specific transporter, the exportin-5, which utilizes nuclear Ran-GTP (65, 66, 20). It is interesting to note that knockdown of the *EXP5* gene, which encodes for exportin-5, produces a reduction of cytoplasmic miRNA molecules without an increase of nuclear ones, suggesting that the transporter may also be involved in pre-miRNA stabilization and protection from nucleases (67).

According to the well-known and accepted processing model, after nuclear export, the pre-miRNA is further processed by the enzyme Dicer, which recognizes the terminal loop and after cleavage releases a small RNA duplex (68). The two strands of these molecules are defined as the guide strand and the passenger strand according to the role they will have on the effector complex for the silencing, which is called RNA-induced silencing complex (RISC; also referred to as the miRISC). After pre-miRNA processing, the RISC-loading complex (RLC), which comprises the Dicer and other molecules,

Table II. *miRNA modulation in thyroid neoplasms. Green boxes indicate down-regulation, red boxes indicate up-regulation and yellow boxes indicate conflicting evidence. Define abbreviated terms.*

miRNA	PTC	PDTC	FTC	ATC	MTC	References
let-7	🟥🟥			🟨🟨		(92, 85, 80, 55)
miR-100	🟥🟥					(92)
miR-107	🟩🟩					(92)
miR-122a	🟥🟥			🟩🟩		(79)
miR-125b	🟥🟥			🟩🟩		(92, 85, 80)
miR-127	🟩🟩				🟥🟥	(93, 79)
miR-128b/b	🟥🟥					(92)
miR-129	🟥🟥					(79)
miR-130b	🟥🟥					(92)
miR-135b	🟩🟩					(92)
miR-137				🟥🟥	🟥🟥	(79)
miR-138	🟩🟩			🟩🟩		(91, 87)
miR-139	🟥🟥					(92)
miR-141	🟥🟥			🟩🟩		(92, 96)
miR-142-3p	🟥🟥					(92)
miR-145	🟩🟩					(92)
miR-146	🟥🟥					(19, 92)
miR-146b	🟥🟥		🟥🟥			(79, 80)
miR-148	🟥🟥					(92)
miR-149	🟩🟩					(92)
miR-154	🟩🟩					(92)
miR-155	🟥🟥		🟥🟥			(79)
miR-15a	🟩🟩					(92)
miR-17-92 cluster				🟥🟥		(86)
miR-181a	🟩🟩					(92)
miR-181b	🟥🟥	🟨🟨				(80)
miR-183	🟥🟥				🟥🟥	(79, 96)
miR-185	🟥🟥					(92)
miR-187				🟥🟥		(79)
miR-191			🟩🟩			(93)
miR-192			🟥🟥			(77)
miR-197			🟥🟥			(77)
miR-200a	🟥🟥			🟩🟩		(92, 96)
miR-200b	🟥🟥			🟩🟩		(92, 96)
miR-200c				🟩🟩		(96)
miR-20a	🟥🟥					(19)
miR-205	🟥🟥			🟥🟥		(79)
miR-21	🟥🟥	🟨🟨			🟥🟥	(80)
miR-211	🟥🟥					(92)
miR-213	🟥🟥					(92)
miR-216	🟥🟥					(92)
miR-218	🟩🟩					(92)
miR-219				🟩🟩		(87)
miR-221	🟥🟥	🟨🟨	🟥🟥	🟥🟥		(19, 80, 94, 79)
miR-222	🟥🟥	🟩🟩	🟥🟥	🟥🟥		(19, 80, 94, 79, 85)
miR-224			🟥🟥	🟥🟥		(79)
miR-26a	🟥🟥	🟩🟩		🟩🟩		(80, 85)
miR-299	🟩🟩					(92)
miR-302b	🟩🟩					(92)
miR-302c	🟩🟩			🟥🟥		(92)
miR-30a-5p	🟥🟥	🟩🟩		🟩🟩		(80, 85)
miR-30d				🟩🟩		(80)
miR-31	🟥🟥					(79)
miR-323	🟩🟩					(92)

Table II. *Continued*

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miRNA	PTC	PDTC	FTC	ATC	MTC	References
miR-328			🟥🟥			(77)
miR-34a	🟥🟥					(92)
miR-34c	🟩🟩					(92)
miR-345				🟥🟥		(87)
miR-346			🟥🟥			(77)
miR-370	🟩🟩				🟥🟥	(92, 95)
miR-375					🟥🟥	(95)
miR-9*					🟩🟩	(95)
miR-96	🟥🟥					(92)
miR-99a	🟥🟥					(92)

PTC: Papillary thyroid carcinoma, PDTC: poorly differentiated thyroid carcinoma; FTC: follicular thyroid carcinoma; UDTC: undifferentiated thyroid carcinoma; MTC: medullary thyroid carcinoma.

catalyses the duplex loading into one of the four Argonauts (AGO) known in humans.

The loading mechanism and the dispensability of proteins that constitute the RLC are still unclear in mammals and contrasting evidence on RISC assembly has been provided (69-71). While in flies and in *C. elegans* the loading process is specific for the miRNA duplex, which is targeted to specific AGO proteins according to its sequence, in humans, a similar system seems not to exist (72). After miRNA duplex loading, the passenger strand is quickly removed and its degradation may be exerted by the AGO2 protein through cleavage, even if cleavage does not seem to be the most frequent mechanism in miRNA processing. It has been shown that AGO proteins are stabilized only when they are bound to miRNA: empty AGO proteins are quickly removed by proteasome-mediated degradation and autophagy.

As soon as the mature, single-stranded miRNA is in place inside the RISC complex, it drives the specificity of target mRNA recognition and subsequent negative regulation. RISC-mRNA interaction is, in fact, elicited through nucleotide pairing between the 'seed' sequence on the miRNA and the corresponding complementary 'seed-match' sequence on its target mRNA.

A limited complementarity (2-9 nt) between these regions is sufficient for a functional interaction that usually lead to a variable degree of mRNA degradation and reduced protein expression. The partial complementarity between seed and seed-match sequences permits miRNAs to interact with several targets. Recent studies showed that non-canonical pathways of miRNA biogenesis exist, as well as Drosha- and DGCR8- independent pathway, terminal uridylyl transferase-dependent (TUTase) pathway and Dicer-independent pathway. One of the first non-canonical pathways was identified and demonstrated with experimental procedures that showed the ability of cells to generate pre-miRNA-like

molecules that bind directly AGOs, including AGO2 that mediates the direct maturation of miRNA (73). An example of Drosha and DGCR8-independent non-canonical pathway is represented by the processing of microRNAs located inside intronic regions (miRtrons) when the introns excised by the spliceosome are directly cleaved and loaded by Dicer. Additionally, recent studies identified miRtrons, like miR-1225 and miR-1228, that are splicing-independent and, accordingly, have been defined simtron (splicing-independent mirtron-like miRNAs) (74). It is important to underline that the functionality of only a small number of these non-canonical miRNAs processing pathways has been demonstrated and that further studies are needed to better understand all the processes involved in miRNA biogenesis. However, it seems clear at this point that the miRNA biogenesis pathway will continue to be a rich source of exciting new discoveries (75).

The identification of miRNAs that may potentially be used as diagnostic and prognostic markers for TC diagnosis and treatment will surely provide valuable means to improve TC management. A considerable degree of observer-related variability exists in morphological diagnosis of TC, especially for certain tumor types like follicular pattern neoplasm. Patient outcome prediction is also biased by this subjective evaluation and enhancements are desirable to improve prognosis determination and therapeutic decision making.

One of the main advantages in the use of miRNAs in practical molecular diagnosis is that these molecules can be easily identified and profiled through minimally invasive processes. The active population of mature miRNAs is very resistant to post-sampling procedures minimizing the risk of pre-analytical artifacts that may be related to RNA degradation. Moreover, it has been shown that the concentration of specific circulating miRNAs in blood is tightly linked to molecular events occurring in those body regions affected by the disease, thus providing an indirect, while easy to use, way of measuring molecular events of diagnostic importance. It was reported recently that a miRNA-based signature can be used to discriminate benign from malignant thyroid nodules using fine-needle aspiration (FNA) samples. The identification of TC-related miRNAs could also serve to support the discovery of potential therapeutic targets and the development of more efficient therapies.

Many studies have been performed to clarify the role of aberrant miRNA functions in TCs and most authors analyzed miRNA expression comparing different forms of TCs in order to identify unique expression patterns (Table II).

In a Chinese study (58), 51 thyroid tumor and adjacent normal tissues were profiled for the identification of miRNA signatures that discriminate between PTCs and nodular goiters (NG). The authors showed that three miRNAs were significantly associated with PTC: miR-30a-3p, miR-146b and miR199b-5p and, while the first has been found to be

down-regulated, the others were up-regulated in tumor tissues. It is obvious that the importance of these profiles in discriminating tissues is limited to comparisons of the same type. Some authors (18), for instance, reported a detailed signature of miRNAs differentially modulated between PTCs and normal tissues that do not include the three miRNAs listed above. They also highlighted the importance of miR-221 using experimental models to evaluate the effects of its over expression. The same authors also investigated the expression of miRNAs in ATC and observed that miR-30d, miR-125b, miR 26a, and miR-30a-5p are drastically down-regulated in tumor tissues. Cantara and co-workers screened miRNA expression in sera from PTC, NG and healthy subjects (76). The analysis revealed eight down-regulated (579, -95, -29b, 5-01-3p, -548d-5p) and three up-regulated (190, -362-3p, -518a-5p) miRNAs in PTC when compared to NG and healthy subjects. After a confirmatory analysis on a wider cohort, miRNAs 579, 95, 29b and 190 maintained their significative differences among sample types. The analysis of 45 primary thyroid samples, comprising FTC samples, follicular adenomas and normal control thyroids (77), resulted in the identification of two miRNAs significantly over expressed in FTC: miR-197 and miR-346. The *in vitro* over expression of both molecules induced proliferation, while their inhibition reduced cellular growth, suggesting an oncogenic role for these miRNAs. In a subsequent study (78), some authors used a more focused technology to characterize the expression of a smaller set of miRNAs in a group of surgically removed thyroid neoplastic and normal samples and in 62 FNA samples. They identified a number of molecules that were specifically modulated in PTC, FTC and ATC. Interestingly, it has been shown that while some miRNAs are usually over expressed in TC (*e.g.* miR-121 and miR-122), the level of their up-regulation is typical of the pathological phenotype considered. A number of studies also focused on miRNA profiling in PDTCs (78-80). It has been shown, for example, that miR-150 and miR-23b can be used to classify conventional and oncocytic PDTC when compared to WDTC (79). Moreover, miR-187, -221, -129, -222, -146b, -339 and -183 (78) were significantly over expressed in PDTCs when compared to normal tissues or hyperplastic nodules.

As mentioned above (1), ATC is likely to arise from PTC and FTC progression. This process requires multiple changes in global gene expression and some of the involved molecular pathways have been already characterized (81-83). According to their role as master gene regulators, the analysis of miRNA expression profiles in ATC has often been investigated in order to elucidate the processes leading to this transition. Even though a number of studies have been performed and reported in the literature, ATC is a very rare disease. For this reason, the data produced so far often lack a strong numeric basis to statistically support the conclusions drawn by

Table III. *miRNA expression profiles associated with specific pathological features. Green boxes indicate down-regulation, while red boxes indicate up-regulation.*

miRNA	Aggressive phenotype	BRAF mutation	Extrathyroidal extension	LN metastasis	Multifocal cancer	Relapse-free survival	Tumor-specific survival	Size ≥ 2 cm	TNM stage I/II vs III/IV
hsa-let-7e					red				
hsa-miR-130b	green								
hsa-miR-146b	red	red							
hsa-miR-151-5p								red	
hsa-miR-181	red		red	red		red		red	
hsa-miR-199b-5p									
hsa-miR-203		red							
hsa-miR-21*		red		red					
hsa-miR-221		red		red				red	red
hsa-miR-222	red	red		red					red
hsa-miR-23b						green			
hsa-miR-34b	green								
hsa-miR-99b						red			
hsa-miR151-5p							red		
hsa-miR-150							green		

authors. In 2007, Visone and co-workers investigated the genome-wide miRNA expression in 10 human ATCs and compared it with 10 normal thyroid profiles. While they proposed a miRNA signature associated with ATC, they also showed that according to functional studies *in vitro*, miR125b and miR-26a down-modulation may be critical for thyroid carcinogenesis (84). Another study (78) indicate that miR-302c, -205 and -137 are over expressed in ATC samples when compared to hyperplastic nodules. Different miRNA signatures have been proposed to discriminate ATC from other TCs. It has been shown (85, 86) that the down-regulation of miR-138 may be sufficient to distinguish ATC from PTC. It was also recently suggested, also, that the up-regulation of miR-200 and mR-30 family members can efficiently discriminate ATC from PTC and FTC.

MicroRNA for Risk Assessment and TC Prognosis

Many efforts have been made for the identification of molecular biomarkers that can be easily used for diagnosis, prognosis and risk assessment. As previously reported, miRNAs have been widely studied and sometimes unequivocally associated with specific types of TCs. Scientific works that aim to reveal associations between miRNA-altered expression and clinically relevant features in PTC are not so numerous but the proposed evidence deserves special attention for their practical relevance. Table III summarizes the involvement of miRNAs in these phenomena according to literature published so far.

An observational study was conducted to identify possible associations between deregulated miRNA expression and clinicopathological features, including *BRAF* mutational status, in a Chinese cohort of 52 patients with PTC (87). It was demonstrated that four miRNAs (221, 222, 146b and 181) had significantly higher expression levels in patients with *BRAF* mutations. The transcript levels of miRNA- 221 and miRNA-181 were also higher in patients with tumor diameter ≥2 cm, while over expression of miRNA-221 and miRNA-222 was observed in patients with advanced TNM stage and lymph node metastasis. Another Asian study (88) complemented these findings with the identification of a significantly relevant association between miR-21* and miR-203 expression and some of the above mentioned features (*BRAF* mutations, TNM stage and occurrence of lymph node metastases). Increased expression of miR-199b-5p in PTC was also shown to be associated with extrathyroidal extension and lymph node metastasis (58).

The opportunity to use circulating miRNAs as biomarkers for minimally invasive molecular tests has been also explored (89) and these small non-coding molecules demonstrated to be predictive of features, like tumor size, multifocality, lymph node metastasis and TNM staging. These molecules, in fact, are very stable in sera and miRNA profiles obtained after blood sampling are often recognizable and reproducible. The mechanisms underlying miRNA release from tissues into the bloodstream, however, is still unclear. Moreover, sera-isolated miRNAs are actually released by many cells belonging to different body regions not all of which are involved in the pathological process. For these reasons, blood sampling-based techniques must take into account the risks for false- positive (*e.g.* increased miRNA production by disease -unrelated regions) and false- negative (*i.e.* when the event to be monitored is the decrease of tumor-specific miRNA level) occurrences.

The impact of miRNA deregulations in prognosis has been investigated in different TC types, like PTC, follicular variant of papillary thyroid carcinoma (FVPTC) and PDTC (90-91, 79). In FVPTC, for example, increased transcription levels of miR-181 and miR-99b produced a decrease in a 10-year relapse-free survival from ~75% to ~18% for the former and from 60% to near 0% for the latter miRNA. On the contrary, a decrease in miR-23b expression in PDTC is reflected in a reduced relapse-free survival. While these studies underline that miRNA profiles can actually perform better than any known clinical indexes (*e.g.* tumor necrosis, increased mitotic index or convoluted nuclei occurrence) in the identification of adverse outcome, further studies will have to prove their potential diagnostic and predictive clinical value.

Conclusion

TC exhibits a wide range of clinical behaviors comprising indolent to highly aggressive forms. While the identification of genetic injuries, like *BRAF, RAS, RET* and *NTRK1* mutations and rearrangements have greatly aided our understanding of thyroid cell transformation, in many cases a clear distinction between risk categories in specific TC subtypes is far from being achieved. Many studies offered insights into the stepwise neoplastic progression, providing molecular signatures that can be ideally used for clinical management of TC. miRNA profiling adds information of critical importance to the molecular picture represented by gene regulation and thus provides completeness and strength to those signatures. According to the literature cited herein, it is possible to conclude that while miRNA signatures are mature enough for TC- type classification, further evidence is needed to confirm that these molecules can be used as valuable biomarkers for outcome prediction in thyroid neoplastic diseases.

Acknowledgements

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