Epigenetic in medullary thyroid cancer: the role of microRNA in tumorigenesis and prognosis

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Purpose of review
MicroRNAs emerged as pivotal regulators of cell differentiation, growth, and cell death, suggesting their implication in tumorigenesis and prognosis of cancer. In the last decades, knowledge about the alterations of microRNAs in medullary thyroid cancer (MTC) is increasing. In this review, we try to summarize the most relevant findings regarding microRNA dysregulation in MTC.

Recent findings
A literature analysis was performed in MEDLINE for studies published up to August 2020. Comprehensively, at least 27 different microRNAs have been investigated in MTC showing evidence for overexpression or underexpression in comparison with normal thyroid tissue samples, healthy blood controls, or primary tumor site or hereditary form of MTC. We highlight the evidence in favor of a possible use of microRNAs for diagnosis, prognosis and treatment in MTC and their role in MTC pathogenesis.

Summary
This review reveals the emerging complexity of the molecular genetic and epigenetic panorama in MTC. Further studies are needed to confirm and refine the findings on microRNA expression pattern in MTC. Thus, in the future, microRNA analysis could enter in clinical practice and may pave the way to new risk-stratification tools and novel therapeutic approaches for MTC.

Keywords
epigenetic, medullary thyroid cancer, microRNAs, prognosis, tumorigenesis

INTRODUCTION

Epigenetics is defined as stably inherited modulations in the gene expression without modification in DNA sequence [1]. DNA methylation and microRNA (miRNAs) regulation are the most known epigenetic modifications. MiRNAs are small single-stranded noncoding RNAs that play a role in the regulation of biological processes by inhibiting gene expression at posttranscriptional level influencing cell differentiation, growth, and cell death. Generally, one miRNA binds to the 3′-untranslated region of target genes mRNAs and suppresses translation and/or causes mRNA degradation of numerous transcripts through the miRNA-induced silencing complex (miRISC) based on sequence complementarity [2,3]. However, less frequently, miRNAs can upregulate gene expression as well [3]. Up to 50% of all coding genes might be regulated by miRNAs and each gene could be influenced by several miRNAs. Therefore, a lot of research in the last decades has demonstrated that miRNA can play an important role in the pathogenesis and prognosis of cancer, including endocrine tumors [4,5]. Generally speaking, tumor upregulated miRNAs are defined as 'onco-miRNAs', whereas the downregulated ones are considered as 'oncosuppressor-miRNAs'; even so, the final biological function of each miRNA is tissue and context-dependent: functioning as onco-miRNAs or oncosuppressor-miRNAs too, depending on the specific tissue background, in a specific neoplastic environment [6].

MiRNAs have been isolated and quantitatively measured both in tissues and biofluids, such as blood serum/plasma. Circulating miRNAs can derive from passive dispersion from dead cells, from active secretion in exosomes or linked to RNA-binding proteins (RBP) [7,8]. Circulating miRNA degradation was avoided by active secretion in vesicles or by binding to RBP. Blood miRNAs may modify the tumor microenvironment [9]. Furthermore, it has

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KEY POINTS

- MiRNAs emerged as pivotal regulators of cell differentiation, growth, and cell death, suggesting their implication in tumorigenesis and prognosis of cancer.
- Numerous studies have proved miRNAs dysregulation in MTC.
- MiRNAs can be assayed in different types of specimens: blood, cytological, and frozen tissues.
- MiRNAs could represent new stable and reliable prognostic markers in MTC to personalize patient's follow-up and treatment from the diagnosis, in the near future.
- Further studies are needed to validate the current knowledge about miRNAs deregulation in MTC and to recognize the upstream miRNAS gene targets more definitely.

been demonstrated that the miRNA dysregulation in tumor tissue and in the exosomes is similar, indicating circulating miRNAs as new possible cancer markers. Hence, circulating miRNAs emerged as novel biomarkers linked to cancer diagnosis and prognosis [10–12].

Medullary thyroid cancer (MTC) is a rare thyroid cancer originating from parafollicular C-cells producing calcitonin (Ct). MTC represents about 2–3% of all thyroid cancers and it can occur in a sporadic form in about 75% or in a hereditary form in the context of multiple endocrine neoplasia (MEN) types 2a or 2b, caused by germline mutations in the *RET* proto-oncogene [13,14]. MTC is responsible for about 8–13% of all thyroid cancer deaths, and 50% of patients with metastatic disease reach a 5-year overall survival rate [14]. Local spread and lymph node metastases are described in more than 75% of cases at presentation, whereas distant localizations, mainly in the liver, lung and bones, are observed in about 4–17% of patients [15]. Evidence suggests that miRNAs play an important role in thyroid carcinogenesis. In particular in MTC, miRNA dysregulation was depicted as a probable early event in C-cell carcinogenesis [16]. The purpose of this review is to emphasize the role of miRNAs and describe the oncogenic or tumor-suppressor function of miRNAs as well as their clinical utility as diagnostic or prognostic markers in MTC.

MICRORNAS IN MEDULLARY THYROID CANCER BIOLOGY

The expression of a miRNA begins with the transcription by RNA polymerase II into longer and noncoding primary RNA (pri-miRNA) [17]. The pri-miRNA is subsequently elaborated by a complex, consisting of the RNase III enzyme DROSHA and DGCR8 protein, that produces the premiRNA. Exportin-5 (XPO5) translocates the premiRNA into the cytoplasm and then it is cleaved by DICER, producing a short duplex fragment. Then the mature miRNA is charged into miRISC and this complex will suppress translation and/or cause mRNA target degradation [18,19].

Some research proved that miRNAs were significantly dysregulated in MTC [16,20–23].

DICER, *DCGR8* and *XPO5*, three genes involved in miRNA production, resulted overexpressed in MTC tissue carrying RET mutations [24]. In particular, MTC carrying RET codon 634 mutations showed a more evident upregulation in DICER1 and DGCR8 compared with RET wild-type cancers, whereas MTC carrying RAS mutations did not demonstrate significant differences with respect to nonmutated tumors. However, further studies are needed to clarify whether the dysregulation in the expression of enzymes involved in miRNA biogenesis lead to miRNA dysregulation and tumorigenesis.

In Table 1, we summarized the findings of main reports in literature, investigating the miRNA deregulation in MTC. Overexpressed miRNAs in MTC compared with the normal thyroid tissues were miR-9, miR-10a, miR-10a-5p, miR-21, miR-127, miR-154, miR-182, miR-183, miR-224, miR-323, miR-370, miR-375 and miR-4465 [16,22,23,25–28]. On the contrary, miRNAs that were proven to be downregulated in MTC tissues were: miR-129-5p and miR-455, miR-30a-5p, miR-130a-3p and let-7i-5p [22,26▪▪,29]. In a research context, what is interesting to underline is that because of an extremely low amount of C cells in the context of a normal thyroid gland, such comparisons involved MTC versus normal tissues of follicular origin: suggesting such miRNAs as new markers of neuroendocrine cancer tumorigenesis.

In addition, Abraham *et al.* found a different expression pattern of miR-9, miR-183 and miR-375 between familial and sporadic MTC cases [21] but these findings were not replicated by other groups [16]. Interestingly, in sporadic cases, miR-127 was upregulated in cancer samples carrying a wild-type *RET* compared with the mutated ones [16].

MiR-21 is a widely investigated onco-miRNA that is upregulated in several human cancers [30▪,31]. Recent studies have shown that miR-21 promotes cell transformation by repressing tumor suppressor genes, such as *PTEN*, *PDCD4*, *RECK* and *TPM1* [32]. We confirmed in two different MTC series, a significant loss of nuclear PDCD4 protein

Table 1. Comprehensive review of microRNA deregulation in medullary thyroid cancer

MicroRNA	Expression	Comparison	References
miR-7	↓	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-9	↑	MTC tissue vs. normal thyroid tissue	[16]
	↓	Sporadic MTC tissue vs. hereditary MTC tissue	[21]
miR-10a	↑	MTC tissue vs. normal thyroid tissue	[22]
	↓	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-10a-5p	↑	MTC tissue vs. normal thyroid tissue	[26▪▪]
miR-21	↑	MTC tissue vs. normal thyroid tissue	[16,23]
miR-29c	↓	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-30a-5p	↓	MTC tissue vs. normal thyroid tissue	[26▪▪]
miR-127	↑	MTC tissue vs. normal thyroid tissue	[16]
	↑	Sporadic MTC tissue wild-type RET vs. sporadic MTC RET mutated	[16]
miR-129-5p	↓	MTC tissue vs. normal thyroid tissue	[29]
miR-130a	↑	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-130a-3p	↓	MTC tissue vs. normal thyroid tissue	[26▪▪]
miR-138	↑	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-154	↑	MTC tissue vs. normal thyroid tissue	[16]
miR-182	↑	MTC tissue vs. normal thyroid tissue	[27]
miR-183	↑	MTC tissue vs. normal thyroid tissue	[16]
	↑	Sporadic MTC tissue vs. hereditary MTC tissue	[21]
miR-193a-3p	↑	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-200b	↓	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-200c	↓	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-224	↑	MTC tissue vs. normal thyroid tissue	[16,25]
miR-323	↑	MTC tissue vs. normal thyroid tissue	[16]
miR-370	↑	MTC tissue vs. normal thyroid tissue	[16]
miR-373	↑	Metastatic MTC tissue vs. primary MTC tissue	[20]
miR-375	↑	MTC tissue vs. normal thyroid tissue	[16,26▪▪,28]
	↑	Plasma MTC vs. plasma healthy controls samples	[26▪▪]
	↑	Sporadic MTC tissue vs. hereditary MTC tissue	[21]
miR-4465	↑	MTC tissue vs. normal thyroid tissue	[26▪▪]
miR-455	↓	MTC tissue vs. normal thyroid tissue	[22]
miR-498	↑	Metastatic MTC tissue vs. primary MTC tissue	[20]
let-7i-5p	↓	MTC tissue vs. normal thyroid tissue	[26▪▪]

MTC, medullary thyroid cancer; RET, rearranged during transfection.

together with miR-21 upregulation, see Fig. 1 [16,23]. The close inverse correlation between these two molecular markers suggests that PDCD4 is a significant target of miR-21 also in MTC, as demonstrated in other mammalian cancers.

MiR-182 is a member of the miR-183 cluster and it was found overexpressed in MTC tissue samples from a cohort of patient samples harboring the aggressive *RET*-M918T mutation [27]. In the in-vitro part of this study, the authors showed that miR-182 is activated by highly aggressive *RET*-M918T and C634W mutations in a NF-κB-dependent manner; the upregulation of miR-182 inhibits *HES1*, which in turn downregulates the Notch1 pathway, promoting an aggressive cell behavior.

Hudson *et al.* reported an overexpression of miR-10a and miR-375 in MTCs' tissues together with a concomitant downregulation of the growth inhibitor Yes-associated protein 1 (YAP1) and MCT8 (a transporter of thyroid hormone), which were identified as potential important downstream targets of miR-375 [22]. Our group confirmed the connection between miR-375 overexpression and YAP1 nuclear loss in a larger cohort of MTCs, suggesting that miR-375 is a negative regulator of YAP1 expression; remarkably, no overlap was found between the

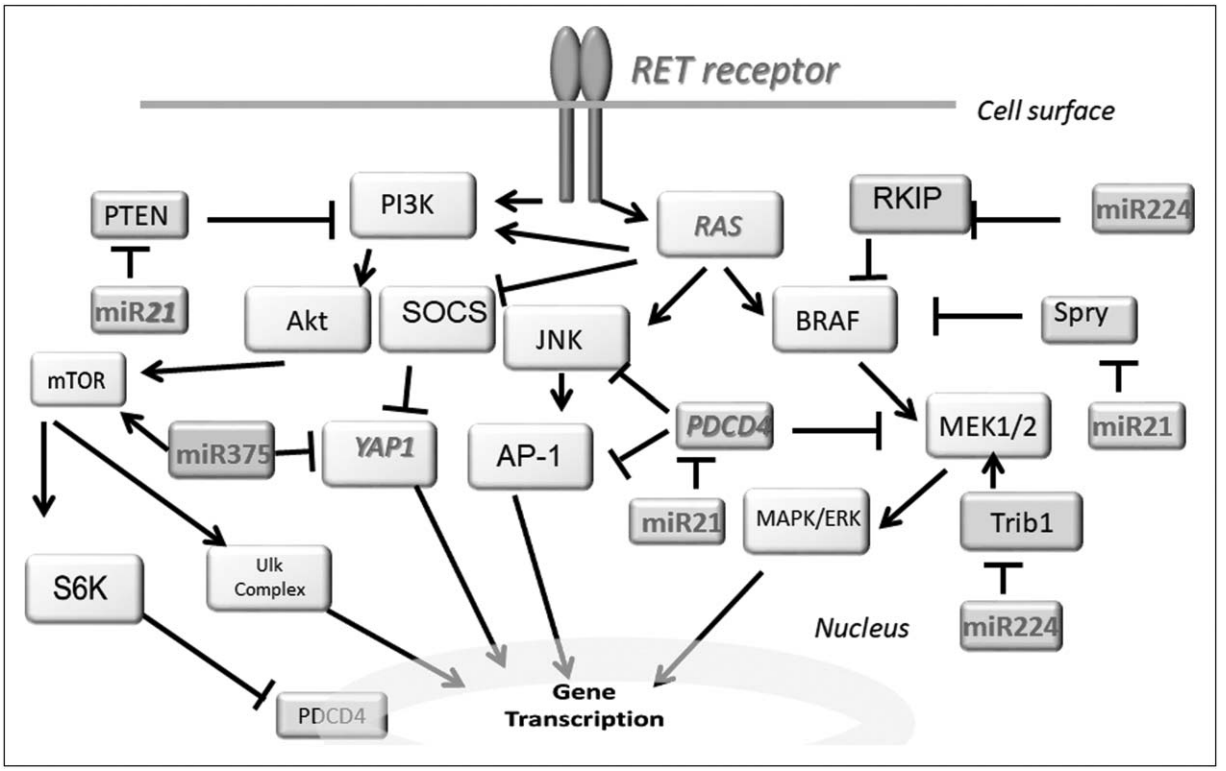

FIGURE 1. Principal microRNAs dysregulation pathways observed in medullary thyroid cancer.

miR-375 overexpression in cancer samples and normal thyroid tissues, see Fig. 1. [28]. MiR-375 overexpression was also reported in hyperplastic C-cells, albeit at a lower level than in MTC tissue, suggesting its dysregulation as an early event in MTC tumorigenesis [26▪▪]. MiR-129-5p seems to behave as an onco-suppressor miRNA, through targeting RET in MTC cells. It has been demonstrated that an overexpression of miR-129-5p leads to a reduced cellular invasion and migration and inhibition of AKT phosphorylation of in-vitro culture of MTC [29]. MiR-183 has been found to repress the expression of *PDCD4* in other tumors [33] and an in-vitro study showed that the knock down of miR-183 expression in human MTC cells produced a significant decrease in the number of viable cells [21].

Another study analyzed the miRNA expression profile in MTC metastases compared with primary cancers [20]. They found an overexpression of miR-130a, miR-138, miR-193a, miR-373 and miR-498 and a downregulation of miR-7, miR-10a, miR-29c and miR-200b/-200c. Indeed, the miR-200 family targeted epithelial–mesenchymal–transition transcription factors like ZEB1, ZEB2, SNAI1, SNAI2 and TWIST-1, bringing to the downregulation of E-cadherin and the upregulation of vimentin, TGFβ1 and TGFβ2, and so enhancing the in-vitro invasiveness of MTC cells [20]. In-vitro studies showed that miR-7 influences the expression of the epidermal growth factor receptor, which is overexpressed in MTC and could contribute to the regulation of the RET pathway [34]. Lastly, miR-29c has been demonstrated to induce apoptosis, upregulating p53 in a direct manner but also indirectly through suppression of p53-negative regulator p85 alpha (the regulatory subunit of PI3 kinase) and CDC42 (a Rho family GTPase) [35].

Nikiforova *et al.* initially investigated miRNA expression profile in two fine needle aspiration biopsy (FNAB) specimens obtained from MTC. In their seminal work, the authors found an upregulation of 10 miRNAs (miR-323, miR-370, miR-129, miR-137, miR-10a, miR-124a, miR-224, miR-127, miR-9, miR-154) with virtually no overlap between MTCs and the other thyroid tumors [36]. These findings are intriguing from a diagnostic point of view as a cytological diagnosis of MTC (using FNAB) is not easily achievable, particularly in the absence of substantial diagnostic clinical data (e.g. an increase in serum Ct). In this setting, miRNAs expression analysis might prove to be an innovative and promising tool in the pathologist's hands, to improve the diagnostic accuracy of classical cytological assessment. Furthermore, in a very recent article by Romeo and colleagues, plasma miR-375 emerged as a new promising diagnostic tool in a small series of advanced MTC (recurrent or persistent) [26▪▪]. In fact, circulating miR-375 levels were significantly

higher in plasma samples of MTC patients in comparison with those from healthy individuals, and were able to distinguish between patients in remission and those with recurrent or persistent MTC.

However, to date, miRNAs do not have sufficient validated data to be used as a diagnostic tool in the everyday clinical practice.

Nevertheless, further studies with reliable findings on large cohorts of MTC are needed to fully understand the different expression pattern of several miRNAs in the biology of familial, sporadic and metastatic MTC; moreover, further understanding about the downstream miRNA target genes is fundamental to get new insights into the MTC tumorigenesis, particularly in case of RET/RAS-negative cancers forms.

PROGNOSTIC MICRORNAS

Given the eminent role of miRNAs in gene expression and cancer tumorigenesis, several studies have proposed that miRNAs could arise as a new prognostic tool in thyroid cancer. As far as MTC is concerned, Ct-doubling times and CEA-doubling times represent the most important biomarkers for predicting the behavior of MTC but these parameters change over time, being longer when the disease is in early stages and shorter in later stages, namely during disease progression [14,37]. Current research is looking for new molecular candidates able to predict from the diagnosis the future course of MTC cancer, with the aim to personalize the patient's follow-up and clinical management, particularly during new systemic therapy approaches.

In this optic, miRNAs could represent new ideal markers thanks to their stability and reliability in different type of specimens (blood, cytological and frozen tissue samples). Abraham *et al.* firstly documented that miR-183 and miR-375 overexpression in MTC were associated with an extensive disease at diagnosis (lateral lymph node metastases) and the worst prognosis during the follow-up in terms of residual disease, distant metastases and mortality [21]. Galuppini *et al.* confirmed that the upregulation of miR-375 in MTC was associated with advanced stages, thyroid capsular infiltration and lymph node involvement at diagnosis and positively correlated with the Ct starting levels [28]. In this study, miR-375 expression levels were also linked to the disease progression; nevertheless, its negative independent predictive potential was not confirmed after multivariate analysis.

The possible negative prognostic marker role of miR-375 in MTC was further validated by Romeo *et al.* in a very recent article where circulating levels of miR-375 were analyzed [26▪▪]. In this study, authors found that plasma miR-375 levels were able to discriminate patients with distant metastasis from those with only locoregional MTC from the start, the former with higher values by comparison with the latter. In their article, circulating miR-375 correlated also with the tumor burden, and within the subgroup of patients with distant metastases, higher circulating levels were associated with worse overall survival (hazard ratio = 10.61). Interestingly, circulating miR-375 values were the only factor that independently predicted a worse prognosis after multivariate survival analysis. So, circulating miR-375 emerged as a new easily quantifiable and obtainable prognostic marker of poor outcome in MTC patients with distant metastases.

Up to now, the only therapeutic options for metastatic MTC patients are locoregional palliative surgeries, external beam radiation therapy, or systemic therapy with tyrosine kinase inhibitors (TKIs), such as vandetanib or cabozantinib. As there are currently no predictive markers available for identifying which patients with advanced progressive MTC might benefit from systemic TKIs, miRNAs have become interesting potential biomarkers. Lassalle *et al.* have showed that in-vitro overexpression of miR-375 is followed by an improved efficacy after vandetanib treatment in MTC cultured cell-lines [38]. Nevertheless, circulating miR-375 levels were evaluated as a possible indicator of eligibility to vandetanib in the study by Romeo and colleagues; unfortunately, miR-375 plasma levels failed to predict the patient's response to vandetanib in this clinical setting but the limited number of partecipants may have influenced the results [26▪▪].

Recently, our group confirmed a previous seminal report on a possible positive prognostic role of miR-224 expression on a large MTC series [16,25]. Higher tissue expression of miR-224 was negatively associated with serum Ct levels at diagnosis and positively associated with the RAS-mutated status. Lower miR-224 values were correlated to an advanced stage disease, lymph node and distant metastases at diagnosis, persistent disease, progressive disease and fatal disease progression with shorter overall survival.

Also tissue miR-21 showed a promising prognostic potential in MTC. Pennelli *et al.* found miR-21 significantly overexpressed in cases of MTC with higher levels of Ct at diagnosis, lymph node metastases, higher stages of disease at diagnosis and patients who were not biochemically cured [23]. In a more recent article, Aubert and colleagues confirmed that miR-183 and miR-21 were associated with lymph node metastases in sporadic MTC [39]. However, only miR-21 was positively correlated with cancer size and serum Ct level at diagnosis,

and it remained an independent predictive factor for lymph node metastases after multivariate analysis.

CONCLUSION

In this review, we have documented the crucial role of miRNA dysregulation in the pathogenesis and progression of MTC, and the potential utility in MTC diagnosis and prognosis.

As the modulation of miRNA expression is only one aspect of the complex molecular events associated with MTC development, it is logical to elaborate a more 'holistic' approach in MTC diagnosis, prognosis and treatment. The molecular characterization of sporadic MTC at diagnosis, based on searching for somatic HRAS, KRAS, NRAS or RET mutations, on profiling the miRNA expression, together with the well established clinical and pathological features, will hopefully pave the way to a customized patient management from the onset, in the near future. In addition, the discovery of new miRNAs and more knowledge on their downstream target genes will offer novel insights about novel therapeutic options, aiming to develop specific modulators capable both of inhibiting oncogenic miRNAs by using miRNA antagonists (antimiRs), and/or restoring key proteins functions by introducing tumor suppressor miRNAs.

Due to the rarity of these cancers and the difficulty to carry out studies with large cohorts, further research is needed to validate and implement the current knowledge about miRNA expression pattern in MTC patients' tissues and biofluids. This approach may refine clinical strategies in patient management, and open the way to novel therapeutic options.

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Conflicts of interest

There are no conflicts of interest.

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- ■ of special interest
- ■■ of outstanding interest

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