In this issue: Non-coding RNAs as potential laboratory biomarkers

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MicroRNAs in endocrine tumors

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ABSTRACT

MicroRNAs (miRNAs) are small, protein noncoding RNAs that regulate gene expression post-transcriptionally. Their role is considered to set the gene expression to the optimal level, or in other words to provide "fine tuning" of gene expression. They regulate essential physiological processes such as differentiation, cell growth, apoptosis and their role is known in tumor development too.

At tissue level differential miRNA expression in endocrine disorders including endocrine malignancies has also been reported. A new era of miRNAs-related research started when miRNAs were successfully detected outside of cells, in biofluids, in cell-free environments. Their significant role has been demonstrated in cell-cell communication in tumor biology.

Due to their stability circulating miRNAs can serve as potential biomarkers. In common diseases circulating miRNAs can be potentially proposed as screening biomarkers and they are also useful to detect tumor recurrence hence they can be applied in post-surgery follow-up too. MiRNAs as diagnostic markers can also be helpful at tissue level when certain

histology diagnosis is challenging. Beside diagnosis, tissue miRNAs have the potential to predict prognosis.

Intensive research is carried out regarding endocrine tumors as well in terms of miRNAs. However, until now miRNAs as biomarkers do not applied in routine diagnostics, probably due to the challenging preanalytics. In this review we summarized tissue and circulating miRNAs found in thyroid, adrenal, pituitary and neuroendocrine tumors. We aimed to highlight the most important, selected miRNAs with potential diagnostic and prognostic value both in tissue and circulation. Common miRNAs across different endocrine neoplasms are summarized and miRNAs enriched at 14q31 locus are also highlighted suggesting their general role in tumorigenesis of endocrine glands.

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INTRODUCTION

MicroRNAs (miRNAs) are small, protein noncoding RNAs that regulate gene expression post-transcriptionally. Through RNA interference miRNAs target mRNAs mainly at 3' untranslated regions but even the coding sequence or 5'UTR were described to be miRNA target regions [1]. After biogenesis the mature miRNA incorporates into miRNA-induced silencing complex (miRISC) [1]. In the miRISC complex, based on sequence complementarity miRNAs cause translational repression, mRNA destabilization or mRNA cleavage. However in some particular cases miRNAs can enhance gene expression as well [1]. It is thought that approximately 30-50% of all protein-coding genes might be controlled by miRNAs [2]. One miRNA potentially targets several transcripts, and one gene's transcription is influenced by numerous miRNAs. Therefore, the net physiological outcome is the result of a miRNA target network. Their role is considered to set the gene expression to the optimal level, or in other words provide "fine tuning" and adaptive setting of gene expression [3]. Their roles have been described in the regulation of several physiological and pathophysiological cellular processes such as proliferation, differentiation, metabolism and apoptosis.

A new era of miRNA-related research started when miRNAs' presence was proved outside of cells, in biofluids. Henceforth extracellular miRNAs have been considered as a novel type of biomarkers that are secreted and can be taken up by various cells. Furthermore, as signal mediators they can function similarly to hormones or cytokines. Several studies showed correlations between circulating miRNA dysregulation and pathophysiological conditions. Regarding neoplastic diseases extracellular miRNAs recently have been investigated and linked to diagnosis, prognosis and recurrence [4]. Differential miRNA expression in endocrine disorders including malignancies has also been reported [5, 6].

Extracellular (EC) miRNAs are secreted by nearly all kinds of cells and therefore they are detectable practically in all body fluids [7]. As unprotected miRNAs are sensitive to degradation mainly through RNAses present in large amount in these fluids, miRNAs in circulation are bound to proteins (mainly Argonaute (AGO)) or they are packaged into vesicles that protect miRNAs against degradation or cleavage. Based on size, EC vesicles are mainly categorized as exosomes, microvesicles or apoptotic bodies. Exosomes are small (approximately 30-100 nm) membrane-limited secreted vesicles [8]. They are formed in the endosomal compartments of cell (multivesicular endosomes or MVEs) and can be released to extracellular space. Microvesicles are more variable in size, typically between 50-1000 nm. They are generated by directly budding or shedding off the plasma membrane. Both exosomes and microvesicles contain various molecules including mRNA, miRNA, proteins, cytokines and different surface receptors

specific for their cell origin. Interestingly, several miRNAs were found in high density lipoprotein (HDL) particles as well [8].

Since their discovery there is exponentially increasing information regarding EC vesicle function. Studies showed their significant role in cell-cell communication in immunology and tumor biology. For instance, exosomes secreted by dendritic cells carry antigens and are able to induce immune response [9]. They can mediate paracrine signals of cancer cells influencing tumor microenvironment by exosome secretion in promoting growth by inhibiting antitumor immune response and by facilitating angiogenesis, cell migration and metastasis [10]. Also, tumor cells were found to exhibit self-promoting effect by secreting microvesicles [11]. However, gastric cancer cells were detected to eliminate tumor-suppressor miRNAs by exosome secretion [12].

On the whole, in a malignant tumor overexpressed (oncogenic) and downregulated (tumor suppressor) miRNAs can be useful as potential biomarkers. MiRNAs are tissue specific, they may be unique identifiers of certain tumor types both in tissue and in circulation. The purpose of miRNA biomarkers can be various and they have a great potential in many ways. In frequent diseases, e.g., thyroid nodule (where prevalence is 2-6%, 19-35% and 8-65% by palpation, ultrasound and autopsy, respectively) circulating miRNAs can be potentially recommended as screening biomarkers [13]. miRNAs as diagnostic markers can also be helpful when cytology following fine-needle aspiration biopsy (FNAB) has to be performed, as from 3-6% to 10-25% of FNAB are interpreted as indeterminate without definitive diagnosis regarding thyroid tumors [13]. Additionally, in any tumor where histological diagnosis can be challenging (e.g. adrenal carcinoma or pheochromocytoma) miRNA biomarkers can be used to help diagnosis. Beside diagnosis, tissue miRNAs have the potential to predict prognosis and therapy response as well. Circulating miRNAs are also useful to detect tumor recurrence hence they can be applied in post-surgery follow-up.

In this review we focus on tissue and circulating miRNAs in thyroid, adrenal, pituitary and neuroendocrine tumors. Although the frame of this review cannot allow assessing all miRNAs in all the above mentioned neoplasms, for that many excellent reviews are available targeting a single tumor type, rather we would like to highlight the most important, selected miRNAs as potential diagnostic and prognostic tissue and circulating biomarkers.

THYROID TUMORS

Thyroid cancer is the most frequent endocrine malignancy. The majority of thyroid tumors (~95%) arise from follicular cells and they are categorized as papillary (PTC, 75-80%) and follicular (FTC, 10-15%) or anaplastic thyroid cancer (ATC, 0.2-2%). Tumors developing from parafollicular C cells are called medullary thyroid cancer (MTC) representing ~5-10% of all thyroid cancers. Although commonly they occur sporadically, some of them (25-30%) are hereditary and part of multiple endocrine neoplasia type 2 (MEN2), caused by germline mutations of the RET proto-oncogene [14]. Most of the well differentiated thyroid cancers (DTC, including PTC, FTC) have excellent prognosis, however patients with ATC have 6-12 months' median survival [13]. Thyroglobulin is widely applied as tumor marker for tumors arising from follicular cells. It is used for evaluation of tumor residuum or recurrence in patients treated by total thyroidectomy and/or radioidine ablation. On the other side calcitonin, a product of parafollicular C cells, is used for the diagnosis and follow-up of MTC. Both tumor markers have limitations therefore miRNAs can be practical in these diseases too [15].

Diagnostic miRNAs

Interestingly, global miRNA downregulation was detected in malignant thyroid cancer compared to normal tissue together with decreased DICER gene expression that was associated with aggressive features [13, 15].

Since the first publication on miRNAs in thyroid cancer in 2005, numerous studies have been

Table 1 miRNAs discriminating benign vs. malignant thyroid lesions from FNAB samples

Authors	Panel of markers	Groups	n (sample number)	Sensitivity (%)	Specificity (%)	Diagnostic accuracy (%)
Stokowy *et al*. 2016	miR-484, miR-184b-3p	mutation-negative follicular thyroid carcinomas and follicular thyroid adenomas	44	89	87	na
Paskas *et al*. 2015	BRAF V600E, miR-221, miR-222, galectin-3	benign vs. malignant thyroid nodule	120	73.5	89.8	75.7
Panebianco *et al*. 2015	miR-146b, miR-222, KIT, TC1	benign vs. malignant thyroid nodule	118	92.3	94.7	96
Shen *et al*. 2012	miR-146b, miR-221, miR-187, miR-30d	benign vs. malignant thyroid nodule	68	88.9	78.3	85.3
Keutgen *et al*. 2012	miR-328, miR-222, miR-21, miR-197	benign vs. malignant thyroid lesions	72	100	86	90
Mazeh *et al*. 2012	miR-21, miR-31, miR-146b, miR-187, miR-221, miR-222	benign vs. malignant thyroid nodule	11	88	100	90

published and extensively reviewed e.g., by Malek *et al.* and Celano *et al.* in 2017. Among several miRNA alterations described, recent meta-analyses emphasize the role of couple of commonly upregulated miRNAs: miR-146b, miR-221, miR-222, miR-181b in PTC compared to normal tissue [13, 15]. Regarding the role of some of these miRNAs, downregulation of KIT was identified as the common potential regulating mechanism [16]. In FTC, miR-637, miR-181c-3p, miR-206, and miR-7-5p were discovered as de novo potential FTC markers based on another meta-analysis comprising 3 independent datasets [17]. In anaplastic thyroid cancer, similarly to DTC cases, overexpression of miR-146b, miR-221, and miR-222 was described together with downregulation of miR-200 and miR-30 families leading to enhanced epithelial-to-mesenchymal transition (EMT) [18]. miRNAs have been dysregulated in MTC too, and this dysregulation was described as a probable early event in C-cell carcinogenesis [19]. Similar to ATC, the underexpressed miR-200 family through regulating E-cadherin by directly targeting ZEB1 (zinc finger E-box binding homeobox 1) and (ZEB2 zinc finger E-box binding homeobox 2) leads to enhanced expression of transforming growth factor β (TGFβ)-2 and TGFβ-1 [13, 15].

Apart from tissue samples fine needle aspiration biopsy (FNAB) specimens were also subjected to miRNA analysis and yielded highly comparable results [15]. Meta-analyses showed that multiple miRNA assays showed a higher diagnostic accuracy than single miRNA and the test results indicated 77% sensitivity, 75% specificity with 0.83 AUC in a receiver operating characteristic analysis [20]. However, several other studies reported promising sets of miRNAs in discriminating benign vs. malignant thyroid lesions from FNAB samples (Table 1).

Circulating miRNAs in both serum and plasma were tested and excellently summarized by Celano *et al*. Although the summary reported a highly variable miRNA expression [15], some miRNAs were identified by multiple groups. In PTC patients, the levels of miR-146b, miR-221 and miR-222 were detected to be higher compared to controls [13, 15, 21]. Post-surgically, significantly reduced miR-151 and miR-222 expression was reported compared to pre-operative samples in more than one study [21]. Also, miR-146b reportedly discriminated benign and malignant tumors with 61.4% sensitivity and 74.3% specificity, while miR-155 had 57.9% sensitivity and 63.2% specificity in serum/plasma [13, 15]. The levels of these two miRNAs were also correlated with lymph node metastases and tumor size [13, 15].

Plasma exosomal miRNAs were also assessed and miR-31 was found to be over-represented in the samples of patients with PTC compared to benign tumors, while miR-21 helped to distinguish between FTC and benign tumors. Using both miR-21 and miR-181a-5p helped to distinguish between PTC and FTC with 100 % sensitivity and 77 % specificity [23].

Prognostic miRNAs

Several studies investigated the potential prognostic value of miRNAs that was summarized in Celano *et al*. Higher expression of miR-146b, miR-221 and miR-222 showed association with prognostic parameters on tissue level [15]. The expression of these miRNAs showed association with tumor size, capsular and vascular invasion, extra-thyroidal extension, lymph node metastases and TNM stage [15]. Besides, the overexpression of miR-146b correlated with multifocality and miR-221 with distant metastases as well in PTC. Overall survival was significantly decreased in patients with higher miR-146b expression in tumor tissue [24]. In a recent study 7 miRNAs (miR-146b, miR-184, miR-767, miR-6730, miR-6860, miR-196a-2 and miR-509-3) were associated with the overall survival and miR-184, miR-146b, miR-509-3 and lysophosphatidic acid receptor 5

(LPAR5) were identified as independent risk factors for prognosis by multivariate analysis [25]. In FTC, the level of miR-221-3p, miR-222-3p, miR-222-5p, miR-10b and miR-92a was higher in metastatic cases vs. non-metastatic. Regarding MTC, Abraham *et al.* published that miR-183 and miR-375 were associated with lymph node metastases, distant metastases and mortality [26]. Also, upregulation of miR-224 was described as potential prognostic biomarker associated with a better outcome in MTC patients [19].

Among circulating miRNAs the level of miR-222, miR-221, miR-146b and miR-151-5p was described to be decreased after tumor removal [15]. Serum level of miR-146a-5p and miR-221-3p was found to be consistent with response to therapy, including patients with structural evidence of disease whose thyreoglobulin (Tg) test remained negative [13, 15]. Therefore it is suggested that these two miRNAs could be applied as post-treatment monitoring biomarker of PTC patients, especially when Tg assay results are uninformative [13, 15].

ADRENAL TUMORS

Adrenal tumors can develop from the adrenal cortex or the medulla. Adrenocortical tumors (ACT) are common and their prevalence reaches 6% after the age of 60 years [27]. Most of them are benign (adrenocortical adenomas, ACA) in nature. Although the most common tumors, so called incidentalomas are non-functioning, some are associated with hormone overproduction syndromes (such as Cushing's, Conn's syndrome, hyperandrogenism). Adrenocortical carcinomas (ACC) however, are rare and aggressive tumors (0.5–2/million per year) [27] with a dismal prognosis where surgical resection is the only curative treatment. The differential diagnosis between ACA and localized ACC can be challenging as radiological and pathological features can be similar but the distinction between them is essential due to the completely different therapy.

HORMONE PRODUCING ADRENAL ADENOMAS

Regarding functional adenomas, peripheral blood hormone testing can help in diagnosis. In aldosterone producing adenomas (APAs) high expression of miR-23 and miR-34a was described [27]. He *et al*. reported 31 miRNAs which were significantly differentially expressed in APAs when compared to normal adrenal cortex. Of these, 23 were downregulated with miR-375 being the most underexpressed [28]. Between expression of miR-375 and miR-7 a strong positive correlation was found indicating a potential synergistic function [28]. MiR-375 substitution in NCI-H295R ACC cell line resulted in decreased cell growth and it inhibited its target gene metadherin (MTDH). Also, miR-375 expression was negatively correlated with APA tumor size reflecting its potential role as a prognostic marker. In cortisol producing adenoma and carcinoma, circulating, extracellular, vesicle-associated miR-22-3p, miR-27a-3p and miR-320b were significantly overrepresented compared to non-functional adenomas [29].

ADRENOCORTICAL CARCINOMAS (ACC)

Diagnostic miRNAs in ACC

Similarly to thyroid tumors, numerous publications reported differentially expressed miRNA profile in ACA and ACC which are exceedingly summarized by Igaz *et al*., Cherradi *et al*., and Hassan *et al*. [30–32]. The most frequently reported overexpressed miRNAs were miR-483-5p and -3p, miR-503, miR-210 and miR-184 in ACC vs ACA. Certainly, miR-483-5p and miR-483-3p have a significant role in adrenal tumorigenesis, its gene is located in the intron of the insulin like growth factor 2 (IGF2) gene, a well-known overexpressed

gene in ACC. Expectedly, a positive correlation was described between miR-483-5p and IGF2 expression levels [33]. Experiments, however, demonstrated that miR-483-5p has an independent role in ACC's pathogenesis as IGF2 transgenic animals did not develop tumors [31]. Indeed, downregulation of miR-483-5p (and miR-483-3p) led to decreased proliferation of ACC cell line [34], and it protected cells from apoptosis by targeting proapoptotic PUMA (p53-upregulated modulator of apoptosis or BBC3: BCL2 binding component 3) [30]. Wang *et al*, suggested the use of the combination of SMAD4 (SMAD family member 4) negative/low expression with elevated miR-483-3p expression that provided a diagnostic specificity of 92.8% for distinguishing ACA vs. ACC (while SMAD4 expression itself demonstrated high sensitivity of 92%) [35]. The overexpression of miR-210 has been also documented by several publications [31, 32]. This miRNA is widely overexpressed in different tumors and it is also called as a "hypoxamir" due to its upregulation by both hypoxia inducible factor 1 subunit alpha and beta (HIF1α, HIF1β). It has a role in tumorigenesis through regulating arrest of cell proliferation, repression of mitochondrial respiration, arrest of DNA repair, vascular biology, and angiogenesis [36]. Among downregulated miRNAs in ACC miR-195 has been frequently reported [31, 32]. As a member of miR-15/16/195/424/497/6838 family it promotes apoptosis together with inhibiting cell proliferation in ACC and other cells targeting cell cycle regulators such as cyclin D1 (CCND1) or cyclin dependent kinase 6 (CDK6) [31].

Beside adrenocortical tumor tissues, miR-483-5p was found to be increased in serum, plasma and circulating exosomes derived from patients with ACC compared to patients with ACA [31, 32]. Circulating miR-100, miR-181b, miR-184, miR-210 and miR-34a were found to be upregulated and miR-195, miR-335, miR-376a downregulated in ACC samples compared to ACA [31, 32]. After tumor removal, the initially highly expressed miR-483-5p decreased and low level of miR-195-5p increased in blood stream of patients with ACC. Therefore, it is suggested that these miRNAs directly derived from ACC tissues [32]. Interestingly, opposite expression of miRNAs in tissue vs. serum was also described in independent publications too. While miR-34a was detected increased in serum and decreased in ACC tissues, miR-376a showed the opposite pattern suggesting a potential selecting mechanism of extracellular secretion [31, 32]. Assessing diagnostic potential of miRNAs Chabre *et al.* also investigated miR-195, miR-335 and miR-376a and found that miR-195 showed the highest sensitivity (90,9%) and specificity (100%) in discriminating ACC patients [37].

Prognostic miRNAs in ACC

The decreased expression of miR-195 and miR-497 in ACC was reported to directly regulate TARBP2 (TARBP2 subunit of RISC loading complex) and DICER1 (dicer 1, ribonuclease III) expression in ACC cells therefore contributing to a global downregulation of miRNA expression [32, 34]. Also, a significant overexpression of TARBP2, DICER, and DROSHA (drosha ribonuclease III) in ACC compared with ACA or normal adrenal cortices were found and inhibition of TARBP2 in human ACC cell line resulted in a decreased cell proliferation and induction of apoptosis [38]. Interestingly, low DICER1 expression was also associated with poor clinical outcome in adrenocortical carcinoma [30–32].

Lower expression of miR-483-5p in combination with increased miR-195 level was reported as predictor of poor prognosis in ACC [30–32]. High expression of miR-503 was associated with shorter survival [34], and increased expression of miR-210 with ACC clinicopathologic parameters of aggressiveness and a poor prognosis [39,

40]. In a recent study, three miRNA clusters were identified related to prognosis [40]. "Mi1" and "Mi2" miRNA clusters including 11 upregulated miRNAs located at Xq27.3 and 38 downregulated miRNAs derived from 14q32 locus were associated with better prognosis (C1B molecular group) while "Mi3" miRNA cluster associated with poor prognosis (C1A group).

Using next-generation sequencing expression levels of 6 microRNAs (miR-503-5p, miR-483-3p, miR-450a-5p, miR-210, miR-483-5p and miR-421) predicted malignant/non-malignant status with over 95% accuracy [41]. In this study the best single miRNA for malignancy was miR-483-3p [41]. MiR-139-5p and miR-376a levels significantly increased in aggressive ACC compared with non-aggressive ACC patients in tumor samples but not in circulation [37]. Additionally, high circulating levels of miR-483-5p or low circulating levels of miR-195 were associated with both shorter recurrence-free survival and shorter overall survival in the study of Chabre *et al*. 2013 [37].

PHEOCHROMOCYTOMA-PARAGANGLIOMA

Although the localization is different pheochromocytomas (PCC) and paragangliomas (PGL) arise from the same type of neural crest tissue of the sympathetic and parasympathetic paraganglia [42]. Tumors of the adrenal medulla are called PCCs and neoplasms developing from thoracic, abdominal or pelvic region paraganglia are named as PGLs. It represents a rare disease as its estimated incidence is 1-8 cases per million worldwide annually [42]. PCCs and PGLs are usually benign (10-year overall survival is around ~96%), however 10% of PCC and even 40% of PGL become metastatic resulting in a poorer prognosis (5-year survival below 50%) [42]. Unfortunately, there are neither clear histopathological signs of malignant behavior nor efficient therapy for malignant PCC/PGL. Therefore, investigating miRNAs as potential biomarkers can be useful in this regard. Nonetheless, compared to thyroid and adrenocortical neoplasms miRNAs are not so extensively investigated in PCC/PGL.

More than 30% of the cases are attributed to germline mutation leading to autosomal dominant genetic syndromes such as multiple endocrine neoplasia type 2A and 2B caused by *RET* mutations, von Hippel Lindau syndrome due to *VHL* mutations, neurofibromatosis type 1 with *NF1* mutations or hereditary PG syndromes caused by mutations of succinate dehydrogenase (SDH) genes. Lately, as a result of next-generation sequencing, novel genes, including *KIF1b, PHD2, TMEM127, MAX, FH, MDH2, GOT2* and *SLC25A11* were identified in the pathogenesis of PCC/PGL [43].

Diagnostic miRNAs

miRNA profile in different genetic subtypes is also distinct. Hypoxia induced miR-210 was found overexpressed commonly in pseudohypoxia-associated PCC/PGLs harboring *SDHB* and *VHL* mutations. Upregulation of miR-139-3p, miR-541, miR-765 and miR-133b was described in *VHL* associated tumors, while miR-96 and miR-183 were found to be overexpressed in neoplasms with *SDHB* mutations [30, 44, 45]. NGF (nerve growth factor) treatment *in vitro* in PCC cell line significantly decreased the level of miR-139-3p and miR-210 and led to differentiation raising the role of these two miRNAs in tumor development [46]. Similarly, through targeting ezrin, miR-96 and miR-183 also suppressed cell adhesion and differentiation [30]. In MEN2B associated PCs miR-885-5p was repeatedly found to be overexpressed [30]. miR-885-5p regulates molecules involved in apoptosis (Casp3) and cell cycle (CDK2) [30], and by targeting IGF-binding proteins its role was supposed in *RET* mutation associated PCC/PGL pathogenesis

[30]. Interestingly, miR-137 and miR-382 can be considered as general PCC/PGL markers as they were found overexpressed in most cases except for *MAX* mutation associated tumors [30]. Interestingly, similarly to ACCs, miRNAs coded at 14q32 genomic region (DLK-MEG3 region) was described to be downregulated in *MAX* mutated and a subset of sporadic PC samples as well [42]. The DLK-MEG3 locus was reported to be hypermethylated in approximately 10% of PC samples and the role of downregulated miRNAs located here was also proposed [30].

Prognostic miRNAs

Similarly to ACC, miR-483-5p was found to be upregulated in malignant PCC/PGL in more than one study [30]. Also, the well-known tumor suppressor miR-15a, miR-16 were underexpressed in malignant PCC vs. benign tumors [47]. These miRNAs were downregulated in several neoplasms and they are considered tumor suppressors by targeting BCL2 and CCND1 [30, 47]. Igaz *et al*. described miR-1225-3p overexpressed compared to sporadic non-recurring, MEN2-, VHL-, and NF1-associated PCs [30]. By targeting Notch signaling, the role of miR-1225-3p is considered in PC tumorigenesis as in *in vitro* experiments Notch-1 inhibited proliferation of PC cell [30].

Compared with benign PCCs, miR-101 level was higher in patients with malignant PCCs and the level of miR-101 was higher in *SDHD* mutation associated tumors [48]. In discriminating malignant from benign PCs AUCs for miR-101 in all investigated PCCs samples were 0.79 and 0.77 for non-SDHD mutant samples [48]. Another study by Patterson *et al.* also proposed that miR-483-5p, miR-101, and miR-183 could serve as useful diagnostic markers (AUC: 0.7; 0.78 and 0.82, respectively) for distinguishing malignant from benign PCCs [44].

PITUITARY TUMORS

Pituitary adenomas are among the most frequent intracranial tumors with a high incidence rate, approx. 10–15 % [49]. Although the great majority of pituitary tumors are benign they can lead to significant morbidity through compressing adjacent structures (visual impairment, headache) or by hormonal disturbance (either hypo- or hyperfunction). Of these 95% are sporadic, only the remaining 5% are associated with genetic syndromes such as MEN1, MEN4, Carney complex or McCune-Albright syndrome. Interestingly, miRNAs are an extensively investigated field in pituitary tumors. Since the first study published in 2005 by Bottoni *et al*.[50], more than a hundred hits have appeared for a search with keywords “microrna” AND “pituitary” AND “adenoma” in Pubmed in February, 2019. These studies broadly evaluated not only miRNA signature characteristics of different types of pituitary adenomas but also target genes and miRNA function as well. Accordingly, several reviews summarizing the expression profile and role of tissue miRNAs widely investigated in pituitary adenoma have been published, one of the most recent by Feng *et al*. in 2018 [51] who extensively summarized miRNAs reported in pituitary adenomas dissected by adenoma subtypes. Here, we aim to highlight the roles of miRNAs by their targets’ function. Although the expression profile and role of tissue miRNAs are widely investigated in pituitary adenoma, we lack information about the diagnostic potential of miRNAs present in circulation. Pituitary adenomas are hormone secreting which give an excellent opportunity to monitor tumor growth and function by hormone tests, therefore the role of miRNAs might be less important. However, regarding non-functional pituitary adenomas a blood-based miRNA biomarker could help the diagnosis and patient follow-up after surgery.

Diagnostic miRNAs

Pituitary subtype-specific miRNA profile was described by several studies [51].

In ***GH producing adenomas*** several miRNAs target insulin growth factor binding proteins (IGFBP-3, -6, -7) that regulates organ development and growth [51]. The overexpressed miR-26a has a key role in the pathogenesis of GH-secreting adenoma by directly regulating PI3K/Akt signaling pathway and LEF-1 that is involved in anterior pituitary development [51]. The overexpression of Pituitary Tumor-Transforming Gene 1 (PTTG1) was demonstrated in ~90% of pituitary adenoma independently of adenoma type. A set of downregulated miRNAs (miR-126, miR-381, miR-665, miR-300, miR-381, miR-329) is suggested to contribute to *PTTG1* overexpression as restoring their level *in vitro* led to suppressed viability and proliferation of pituitary cells [52]. High Mobility Group AT-Hook Protein 2 encodes a non-histone chromosomal high mobility group (HMG) protein, as a transcriptional regulator it controls cell cycle and pituitary cell proliferation. Numerous miRNAs were identified targeting HMGA1 and 2 in GH, PRL and non-functioning pituitary adenomas [51]. In sporadic GH-producing adenomas miR-34a and miR-107 were found to regulate *AIP* a well-known tumor suppressor frequently mutated in familial pituitary adenomas.

In ***corticotroph adenomas*** miR-26a was massively overexpressed compared to normal pituitary. MiR-26a downregulated Cyclin A and Cyclin E and it targeted PRKCD (protein kinase C delta) that was reported to suppress ACTH secreting pituitary cells [51].

In ***prolactinomas*** HMGA1 and 2 as a target of several miRNAs were also validated. Interestingly the expression of miR-432 were found to be positively correlated with serum prolactin [51]. The role of downregulated miR-410 was also proved in pathogenesis of prolactinoma by targeting Cyclin B1 [51].

In ***non-functioning pituitary adenomas*** (NFPAs) the role of HMGA1 and 2 proteins also has to be mentioned regulated by several miRNAs [51]. Cell cycle in NFPAs is regulated in G2M transition by miRNAs through targeting Wee1 and CDC25A [53]. The TGFβ signaling is also controlled by miRNAs through SMAD3 [54]. Additionally, miR-106b influenced migration and invasion of pituitary adenoma cells via regulating PTEN and further activity of the PI3K/AKT signaling pathway and MMP-9 expression [51].

In pituitary carcinoma metastasis expression of miR-20a, miR-106b and miR-17-5p were increased compared to the primary neoplasm and these miRNAs were proved to be involved in pituitary carcinoma metastasis by attenuating PTEN and TIMP2 (TIMP metallopeptidase inhibitor 2) [55]. In ACTH producing carcinomas miR-493 was significantly upregulated compared to ACTH adenomas while miR-122 overexpressed in both corticotroph adenomas and carcinomas compared to normal pituitary [56]. LGALS3 and RUNX2 are both predicted targets of miR-493 and these genes have been shown to have roles in pituitary tumor cell growth [56].

Prognostic miRNAs

Level of several miRNAs (miR-24, miR-34a, miR-93, miR-148-3p, miR-152, miR-132, miR-15a, and miR-16) are significantly lower in invasive pituitary adenomas compared with non-invasive ones [57]. In prolactinomas, miR-183 was found downregulated in aggressive tumors and it inhibited tumor cell proliferation by inhibiting KIAA0101 that is involved in cell cycle activation and inhibition of p53-p21 mediated cell cycle arrest [58]. In corticotroph adenomas miR-93-3p, miR-93-5p, miR-25-3p and miR-106b-5p were detected to be overexpressed in invasive tumors compared to non-invasive ones through

targeting *MCM7* the overexpression of these miRNAs led to increased invasiveness and unfavorable outcomes after resection [51, 59]. Patients with corticotroph adenomas with decreased level of miR-141 had higher chance of remission [60].

In NFPAs several miRNAs showed correlation with tumor size some of the (miR-450b-5p, miR-424, miR-503, miR-542-3p, miR-629, and miR-214) were underexpressed and target *SMAD3* [54]. In invasive adenomas expression levels of miR-181b-5p, miR-181d, miR-191-3p, and miR-598 were upregulated, and the expression levels of miR-3676-5p and miR-383 were downregulated [61]. In GH3 cells Caveolin-1 (CAV1) was reported to promote invasion while silencing *CAV1* indirectly induced miR-145, miR-124, and miR-183 that suppressed the migration and invasion of pituitary adenoma cells through targeting *FSCN1, PTTG1IP* and *EZR,* respectively [57].

NEUROENDOCRINE TUMORS (NETs)

Neuroendocrine tumors (NETs) consist of heterogeneous neoplasms of different origin arising from neuroendocrine cells throughout the body (most commonly from the lungs, pancreas, small intestine, and rectum). Gastro-entero-pancreatic NETs (GEP-NETs) represent less than 1% of digestive cancers and 7-21% of all neuroendocrine neoplasms [62]. Lung NETs originate from pulmonary neuroendocrine cells accounting for approximately 25% of primary lung neoplasms. Lung NETs classified into the following subtypes: typical carcinoids (TCs, well differentiated, low-grade); atypical carcinoids (ACs, well-differentiated, intermediate-grade); large cell neuroendocrine carcinomas (LCNECs, poorly differentiated, high-grade); and small cell lung cancer (SCLCs, poorly differentiated, high-grade) [63]. NET as a heterogeneous group have different behavior and prognosis but they express neuroendocrine markers such as chromogranin A (CgA) and synaptophysin. Based on the World Health Organization (WHO) classification the prognosis is characterized by the grade of neuroendocrine differentiation and the proliferative index (Ki-67) from G1-G3. However, guidelines indicate that prognosis is also influenced by several other factors, such as patient age, tumor site, metastatic spread and hormonal production [64]. Unfortunately as more than 80% of patients usually present with metastatic disease, NET prognosis is poor, due to the relative lack of effective therapy [63, 64]. Among circulating biomarkers, CgA has been measured in several NETs, but its value as a prognostic biomarker in NETs is limited [64], therefore identification of prognostic factors to predict outcome would be fundamental.

Diagnostic miRNAs

Interestingly, little information is available regarding miRNA profile in ***gastric NETs (gNETs)***, however it was described that gastrin-induced miR-222 overexpression resulted in reduced p27, which in turn caused actin remodeling and increased migration in human stably CCK2 receptor expressing gastric adenocarcinoma cell line [65].

In circulation, serum miR-222 expression was increased in hypergastrinemic patients with autoimmune atrophic gastritis and type 1 gastric NET. Because its level decreased in patients after CCKR2 agonist treatment miR-222 was proposed to be a promising biomarker for gastrin induced premalignant changes in the stomach [65].

However, different miRNA sets with altered expression were described regarding ***pancreatic NET (pNET)***, comparing functional, nonfunctional NET, normal pancreas, normal pancreatic islets in any combinations that is reviewed in detail by Malczewska *et al*. and Zatelli *et al*.

[64, 65]. The overexpressed miR-103, miR-107 and the underexpressed miR-155 discriminated sporadic pancreatic NET (insulinoma and non-functioning) from acinar cell carcinomas. The role of downregulated miR-155 was suggested in pathogenesis by targeting proapoptotic tumor protein p53 inducible nuclear protein 1 (TP53INP). Also, miR-144/451 cluster and miR-21 was found overexpressed compared to normal pancreatic islets [65]. MiR-204 was found primarily expressed in insulinomas and correlated with immunohistochemical expression of insulin [66]. Interestingly, several miRNAs located at 14q32 region showed dysregulated expression, including miR-144/451 cluster [65]. Interestingly, these miRNAs showed overexpression in insulinomas compared to other endocrine tumors where miRNAs of this region are frequently downregulated. In *in vitro* experiments miR-144 induced cell proliferation in murine pancreatic β cells and regulated Akt signaling by targeting *PTEN* [65]. Additionally, miR-451 also promoted cell proliferation by regulating cell cycle through targeting p19 [67]. Overexpression of miR-21 were reported overexpressed in pNET [65, 66]. Expression of miR-642 correlated with Ki67 (MiB1) score and miR-210 correlated with metastatic disease [68].

13 miRNAs were identified by comparing serum from pNET patients and healthy volunteers and miR-193b was up-regulated in both pNET tissue and serum when compared to controls described by Thorns *et al*. [68]. In addition, miR-1290 showed overexpression in pNETs and the latter could accurately distinguish patients with low-stage pancreatic cancer from healthy controls and subjects with chronic pancreatitis [69].

MiRNA profile of ***small bowel NET (sbNET)*** was also investigated, however fewer experimental information are available regarding miRNA's function studies and target validation. MiR-7-5p, miR-182, miR-183 and miR-96-5p were found to be upregulated in sbNET compared to normal small bowel consequently in different studies [65]. Furthermore, miR-182, miR-183 and miR-96 overexpressed in NET metastases compared to primary tumors [65]. Similarly, upregulation of miR-196a was described in numerous studies however its role in cell proliferation could not be confirmed [65, 69]. In addition, the downregulation of miR-129-5p and miR-133a was also established in sbNET metastases vs. primary tumors [65].

Similarly to tissue, overexpression of miR-182, miR-196a and miR-200a and downregulation of miR-31, miR-129-5p and miR-133a were detected in blood of sbNET patients and the level of some of them changed upon somatostatin treatment [65].

Similarly to sbNET, in ***appendiceal carcinoids*** without metastases low levels of miR-96 and high levels of miR-133a were detected [65]. In ***colorectal NET (cNET)*** patients underexpression of miR-186 was found in tumor tissue, blood and stool samples compared to controls. In parallel, PTTG1 upregulation was detected in the same samples together with decreased miR-186 expression therefore the authors suggested that upregulation of PTTG1 was induced by the loss of miR-186 [70].

Interestingly, based on miRNA expression profile analysis common origin for pulmonary carcinoids and GI-NETs was suggested by Yoshimoto *et al.* [71].

Lung NETs. The expression profiles of pulmonary carcinoids and SCLCs were quite different, indicating the distinct genesis of these neuroendocrine neoplasms [71].

Prognostic miRNAs

A study by Sadanandam *et al*. identified molecular subtypes of ***pNET***s (islet-like, intermediate and metastasis-like primary types) [72]. As these subtypes exhibit distinct metabolic profiles marked by differential pyruvate

metabolism, substantiating the significance of their separate identities they may have role to predict different behavior. Expectedly, the three subtypes have distinct mRNA and miRNA signatures as well [72]. Roldo *et al*. reported the overexpression of miR-21 as positively correlated with Ki-67 proliferation index and presence of liver metastases [66]. Besides, expression of miR-642 was also described to correlate with Ki67 index score while miR-210 correlated with metastatic disease [68]. Additionally miR-196a was identified as prognostic factor in pNET as its expression significantly associated with stage, and mitotic count [64]. Also, high miR-196a level was associated with decreased overall survival and disease-free survival. The hazard ratio for recurrence of patients with high miR-196a expression was 16.267 [73].

In ***sbNET*** increased plasma miR-21 and decreased miR-150-5p were characteristic to metastatic tumors [74]. In line with this, low plasma miR-21 and high miR-150-5p levels were associated with significantly prolonged overall survival [74]

In ***rectal carcinoids*** miR-885-5p was identified as upregulated in tumors with lymphovascular invasion. Also, high miR-885-5p expression was independently associated with lymphovascular invasion. Therefore miR-885-5p is suggested as a potential biomarker for predicting malignancy [75].

Regarding NET in the lung Zatelli *et al*. thoroughly summarized miRNAs with prognostic role [64]. miR-150 and miR-886-3p were found downregulated while miR-92a2* and miR-7 upregulated in SCLC that showed correlation with overall or disease-free survival. The latter miRNAs were found to be associated with chemoresistance too [64]. In other publications including typical, atypical carcinoids and large cell neuroendocrine carcinomas the upregulated miR-21 and the downregulated miR-409-3p, miR-409-5p, and miR-431-5p correlated with the presence of lymph node metastases and set of other 5 upregulated miRNAs with overall survival [64].

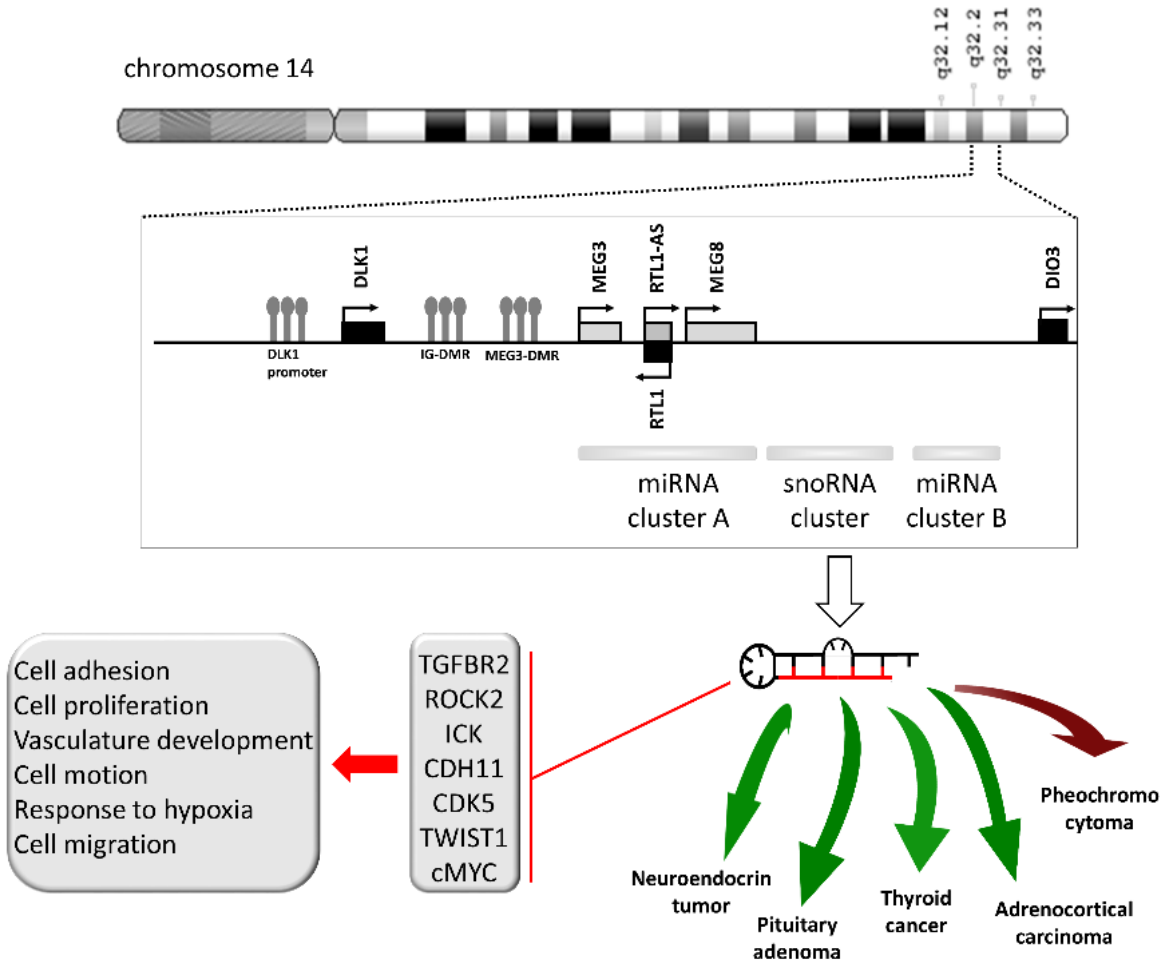
COMMON miRNAs DETECTED IN VARIOUS ENDOCRINE TUMORS

Although miRNAs' expression and their functions are tissue specific, discussing common miRNAs among different endocrine tumors can still be an interesting aspect reflecting potential common pathomechanism. For instance, miR-210 was overexpressed in several endocrine tumors, such as ACC and PCC/PGL [30, 31]. Also, its increased expression seems to be an unfavorable prognostic factor correlating clinicopathologic parameters of aggressiveness and a poor prognosis in ACC and in pNET [39, 40, 68]. Usually, the increased expression of miR-210 might be a consequence of hypoxic environment within tumor tissues or activation of pseudohypoxia signaling pathway in PPC/PGL. As a general phenomenon increase of miR-210 was also described in other, non-endocrine solid tumors (i.e. breast, lung, head and neck, pancreatic and glioblastoma).

Overexpression of miR-483-5p and miR-483-3p in tissues and in circulation (serum, plasma and exosomes) is almost a unique characteristic of ACC and is considered as a prognostic marker [30, 31, 32, 33]. It predicted malignant/non-malignant status and it associated with both shorter recurrence-free survival and shorter overall survival. Interestingly, not only for tumors of adrenal cortex but for tumors originated from adrenal medulla (PCC/PGL) miR-483-5p is also proposed as useful diagnostic markers for malignancy [30].

Two well-known tumor suppressor miRNAs: miR-15a and miR-16 are downregulated in pituitary adenomas and in PCC where they were described to be able to discriminate benign

Figure 1 **miRNAs coded at 14q32 is commonly dysregulated in endocrine neoplasms: downregulated in neuroendocrine tumors, pituitary adenomas, thyroid cancer and adrenocortical carcinoma (indicated by *green arrows*) and overexpressed in pheochromocytomas (showed by *red arrow*)***



**At 14q32 region paternally imprinted genes (DLK1, RTL1, and DIO3) are illustrated by black, maternally imprinted noncoding RNAs (MEG3, anti-RTL1, and MEG8) are represented by grey boxes.*
Lollipops indicate methylation sites at DLK1 promoter region, intergenic differentially methylated region (IG-DMR), and MEG3 differentially methylated region (MEG3-DMR) region. MiRNAs at 14q32 region are located in 2 clusters separated by a small nucleolar RNA (snoRNA) cluster. miRNA cluster 1 contains 10 miRNAs and cluster 2 encodes more than 40 miRNAs. Therefore, alteration in methylation, chromatin remodeling, genomic imprinting imbalance or structural loss can lead to dysregulated miRNA expression. 14q32 miRNAs influence cell proliferation, cell adhesion, and migration through regulating targets involved in transforming growth factor beta signaling or epithelial–mesenchymal transition.

TGFBR2: Transforming Growth Factor Beta Receptor 2; ROCK2: Rho Associated Coiled-Coil Containing Protein Kinase 2; ICK: Intestinal Cell Kinase; CDH11: Cadherin 11; CDK5: Cyclin Dependent Kinase 5; TWIST1: Twist Family BHLH Transcription Factor 1; cMYC: MYC Proto-Oncogene; RTL1: retrotransposon-like gene RTL1; RTL1-AS: retrotransposon-like gene RTL1 antisense; DLK1: Delta Like Non-Canonical Notch Ligand 1; MEG3: Maternally Expressed 3; MEG8: Maternally Expressed 8, Small Nucleolar RNA Host Gene; DIO3: Iodothyronine Deiodinase 3.

and malignant forms of the disease [47, 51]. In addition, the lost expression of general tumor suppressor miRNAs targeting BCL2 and CCND1 have been described in other, non-endocrine tumors as well [30, 47].

Additionally, Pituitary Tumor-Transforming Gene 1 (PTTG1) is another target gene that is regulated by miRNAs in endocrine tumors. Several miRNAs (miR-126, miR-381, miR-665, miR-300, miR-381, miR-329) are suggested to contribute to PTTG1 overexpression in pituitary adenomas [51], that is also upregulated in colorectal NET by the loss of miR-186 [70]. However, PTTG1 overexpression is not unique for endocrine tumors as it was described in several other malignancies with prognostic potential.

MiR-21, a widely investigated oncomiR, has been found up-regulated in many types of human tumors. Indeed, in lung NET and pNET it was described overexpressed and in pNET its expression positively correlated with proliferation index and with metastases [65, 66]. Additionally, its level was reported elevated in circulation in sbNET where it was described to be characteristic to metastatic tumors and associated with significantly prolonged overall survival [74]. In follicular thyroid cancer plasma level of exosomal miR-21 helped to distinguish between FTC and benign tumors [23].

Similarly to miR-21, miR-222 is differentially expressed in several endocrine tumors, such as in gastric NET (where it is induced by gastrin) and in thyroid cancer (both papillary and anaplastic) [13, 15, 18, 65], whereas in follicular thyroid cancer the tissue level of miR-222 was prognostic (higher in metastatic cases vs. non-metastatic). In the blood stream of PTC patients, miR-222 expression significantly decreased after surgery [21].

CONCLUSIONS AND FUTURE PERSPECTIVES

It is obvious that miRNAs are potentially useful and promising tissue and circulating biomarkers. They are extensively investigated in different neoplasms including tumors of the neuroendocrine system. Interestingly, miRNAs coded at 14q32 region seems to be dysregulated in almost all endocrine neoplasms (Figure 1) and several other tumors. This region is frequently found hypermethylated in cancer compared to normal tissues. The downregulation of miRNAs located at 14q32 region shows important correlations with poor prognosis and aggressiveness in different cancer types. Studies also suggest chromatin remodeling by lncRNA-mediated mechanisms in this region beside DNA methylation that may also influence miRNA expression. Additionally, as 14q32 is an imprinted region and imprinting imbalance could also result in alteration of paternally and maternally expressed genes.

Regarding their application to routine diagnostics thyroid tumors and FNAB samples seem to be investigated the most intensively. Although high sensitivity and diagnostic accuracy of miRNAs have been reported in thyroid cancer which may be suitable for differentiation of benign vs malignant thyroid nodules, routine use of miRNAs as biomarkers has not been widespread yet either in thyroid or other endocrine and non-endocrine tumors. In literature it is suggested that this may be due to challenging preanalytics, especially in case of bio-fluids. Additionally, there are no IVD-CE (IVD: applicable for In Vitro Diagnostic, CE: complies with the essential requirements of the relevant European health, safety and environmental protection legislations) marked assays to measure miRNAs in clinical care. That also may delay miRNAs' application as potential diagnostic biomarkers.

However, regarding thyroid nodules there are common miRNAs among different studies discriminating benign and malignant nodules (e.g. miR-221, miR-222, and miR-146b). These miRNAs should be further investigated as potential candidates for clinical use.

In pituitary neoplasms, however there is the most information gathered regarding the pathogenesis and miRNA-regulated networks, there is basically no information on miRNAs in biofluids. It can be a consequence of negative results or the difficulty of sample group homogenization due to the different adenoma groups especially in the light of the 2017 WHO classification. In adrenocortical carcinoma the role of one miRNA (miR-483-5p) has been repeatedly proved by several studies in both tissue level and in circulation. And finally, the prognostic role of miRNAs is also intensively investigated in NETs however, again, due to the heterogeneous tumor group there is still a need for data regarding the application of any miRNA biomarker in clinical use. Behind the discrepant results found in literature not only the tumor heterogeneity but also the differences in study design and technical implementation could be suspected. Regarding circulating biomarkers, currently there are many biological (age, gender, BMI, smoking status, disease stage, other accompanying illnesses, medications, etc.) and technical (serum, plasma, extracellular vesicle as source, different nucleic acid extraction methods, miRNA detection methods, etc.) factors known and unknown which need standardization and harmonization in order to establish evidences before application of miRNAs as biomarkers.

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