Cell. Mol. Life Sci. (2015) 72:417–428
DOI 10.1007/s00018-014-1752-7

Cellular and Molecular Life Sciences

REVIEW

MicroRNAs in adrenal tumors: relevance for pathogenesis, diagnosis, and therapy

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Received: 27 July 2014 / Revised: 16 September 2014 / Accepted: 29 September 2014 / Published online: 9 October 2014
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Abstract Several lines of evidence support the relevance of microRNAs in both adrenocortical and adrenomedullary (pheochromocytomas) tumors. Significantly differentially expressed microRNAs have been described among benign and malignant adrenocortical tumors and different forms of pheochromocytomas that might affect different pathogenic pathways. MicroRNAs can be exploited as markers of malignancy or disease recurrence. Besides tissue microRNAs, novel data show that microRNAs are released in body fluids, and blood-borne microRNAs can be envisaged as minimally invasive markers of malignancy or prognosis. MicroRNAs might even serve as treatment targets that could expand the rather-limited therapeutic repertoire in the field of adrenal tumors. In this review, we present a critical synopsis of the recent observations made in the field of adrenal tumor-associated microRNAs regarding their pathogenic, diagnostic, and potential therapeutic relevance.

Keywords Biomarker · Pathway · Cancer · Treatment

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Introduction

Adrenal tumors are common with an average frequency of 2–3 % in autopsy series [1]. Most of these tumors are clinically silent and incidentally discovered benign adrenocortical tumors [1]. Hormone-producing adrenocortical adenomas (cortisol—Cushing's syndrome, aldosterone—primary aldosteronism) and catecholamine-secreting adrenomedullary pheochromocytomas (PCC), however, result in significant morbidity and mortality. Malignant tumors of the adrenal, adrenocortical cancer (ACC), and malignant pheochromocytoma are rare [2, 3]. The prognosis of ACC is poor in advanced stages: 5-year survival in stage 3 is 50 %, and only 13 % in stage 4 [2]. The five-year survival of malignant PCC varies between 20 and 50 % [3].

The pathogenesis of sporadic adrenocortical tumors is poorly elucidated, and several questions are to be answered in PCC tumorigenesis as well. There are several challenges in the clinical management of these tumors including the lack of reliable preoperative plasma or serum markers of malignancy or recurrence. The histological diagnosis of malignancy in adrenocortical tumors is problematic, but it is not feasible in PCC at all where malignancy can only be established by the appearance of metastases [2–4]. Besides surgical removal, there are few effective medical treatment options for advanced metastatic adrenocortical and adrenomedullary tumors [2–4].

Since the discovery of RNA interference and the subsequent description of microRNAs as their endogenous mediators, a vast array of experimental findings underline the relevance of microRNAs and their pathways in various human tumors [5, 6]. MicroRNAs are non-protein-coding small RNA molecules encoded by distinct genes that undergo a sophisticated maturation process and their mature single-stranded forms specifically bind the 3′ untranslated region of messenger RNAs (mRNA). Cytoplasmic microRNAs inhibit mRNA translation or induce their degradation and thereby constitute a very specific way of posttranscriptional gene expression regulation forming part of the epigenetic machinery [6]. MicroRNAs are involved in the regulation of basic cell biological processes, immune regulation, ontogenesis, etc. [7]. Novel data show that besides posttranscriptional gene regulation exerted by cytoplasmic microRNAs, nuclear microRNAs are also involved in the regulation of gene transcription [8].

Overexpressed microRNAs in tumors relative to normal tissues have been termed oncogenes, whereas underexpressed microRNAs relative to normal tissues are regarded as tumor suppressors following the classical oncogene-tumor suppressor dichotomy [5, 6, 9]. Oncogenic microRNAs might contribute to tumorigenesis by targeting cellular tumor suppressor mRNAs, whereas tumor suppressor microRNAs target oncogenic mRNAs [9]. MicroRNAs act in a tissue-specific manner and therefore the same microRNA can function as a tumor suppressor in one tissue, but also as an oncogene in another [9].

Differentially expressed microRNAs can be exploited as markers of malignancy. This can be especially useful for the diagnosis of tumors, where histological analysis is difficult (e.g., diagnosis of differentiated thyroid tumors) [10]. Since microRNAs are surprisingly stable, their diagnostic applicability is greatly extended by the use of formalin-fixed paraffin-embedded (FFPE) archived tissue blocks [11]. Novel observations have revealed that microRNAs enter body fluids via the secretion of exosomes, microvesicles, or in complex with macromolecules like high-density lipoprotein and might exhibit features of hormones conveying gene expression information [12]. Blood-borne (plasma or serum) microRNAs might have great potential as minimally invasive biomarkers, and there are several data in various tumors supporting their clinical applicability [13]. Apart from their pathogenic and diagnostic relevance, microRNAs might also be envisaged as potential future targets of therapy [14].

Table 1 Validated microRNAs significantly differentially expressed in studies involving ACC samples

Study	Sample source and method of analysis	Sample distribution	Validated microRNAs in ACC relative to adenomas or normal adrenal cortices
Tömböl et al. [24]	Tissue—TLDA	7 ACC, 19 ACA, 10 NA	**miR-503**↑, miR-184↑, **miR-210**↑, miR-214↓, miR-511↓, miR-375↓
Soon et al. [19]	Tissue—microarray	22 ACC, 27 ACA, 4 NA	**miR-195**↓*, **miR-335**↓, **miR-483-5p**↑*
Patterson et al. [18]	Tissue—microarray	10 ACC, 26 ACA, 21 NA‡	miR-100↓, miR-125b↓, **miR-195**↓, **miR-483-5p**↑, **miR-483-3p**↑
Özata et al. [20]	Tissue—microarray	25 ACC, 43 ACA, 10 NA	**miR-483-5p**↑, **miR-483-3p**↑, **miR-210**↑, miR-21↑, miR-1974↓, **miR-195**↓, miR-497↓, **miR-503**↑*, miR-1202↑*, miR-1275↑*
Schmitz et al. [29]	Tissue (FFPE)—TLDA	4 ACC, 9 ACA, 4 NA + validation cohort ($n = 15$)	miR-139-3p↓, miR-675↓, **miR-335**↓
Chabre et al. [31]	Tissue—microarray	12 ACC†, 6 ACA	**miR-195**↓, **miR-335**↓, **miR-483-5p**↑, miR-139-5p↑**, miR-376a↑**
	Serum—qRT-PCR	23 ACC†, 14 ACA, 9 NA	miR-195↓, miR-335↓, **miR-483-5p**↑**
Szabó et al. [62]	Plasma—qRT-PCR	13 ACC, 12 ACA	miR-100↑, miR-181b↑, miR-184↑, miR-210↑, **miR-483-5p**↑
Patel et al. [63]	Serum—qRT-PCR	17 ACC, 22 ACA	miR-34a↑, **miR-483-5p**↑

microRNAs highlighted in *bold* have been validated in at least two independent studies

↓ down-regulation, ↑ up-regulation relative to adenoma or normal adrenal, *ACC* adrenocortical cancer, *ACA* adrenocortical adenoma, *NA* normal adrenal cortex, ‡ NA were pooled in this study, † ACC were subdivided in this study in aggressive and non-aggressive subgroups, * associated with shorter survival, ** Overexpressed in aggressive ACC relative to non-aggressive ACC, *TLDA* TaqMan low-density array, *qRT-PCR* quantitative real-time polymerase chain reaction

The potential of miRNAs in the diagnosis and treatment of adrenal tumors remains to be exploited because of numerous unresolved issues associated with miRNA research. These include differences in microRNA profiling results established by different research groups and numerous technical issues. Different platforms (low-density polymerase chain reaction (PCR)-based arrays, microarrays, next-generation sequencing), statistical approaches, choice of reference genes, etc., might account for these discrepancies in addition to the different composition of patient cohorts. The target mRNAs for microRNAs are most easily identified by in silico target prediction approaches that must be validated experimentally [15]. Results of high-throughput analyses and microRNA-based pathways must also be validated by a different technique (e.g., real-time PCR) [16].

In this review, we present a synopsis of the recent observations made in the field of ACC- and PCC-associated microRNAs highlighting their relevance in tumorigenesis, diagnosis, and potential for therapy.

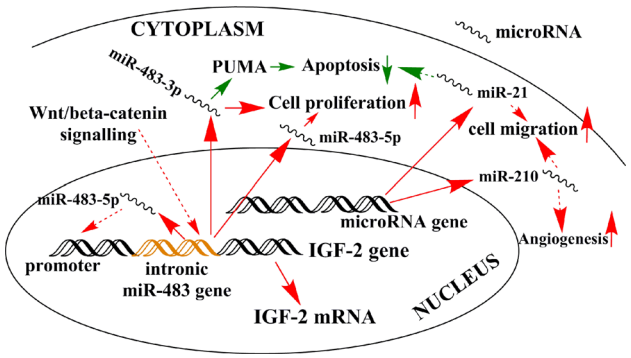

Fig. 1 Schematic representation of the potential relevance of overexpressed microRNAs in ACC pathogenesis. *Continuous arrows* represent validated interactions in ACC, whereas *dashed arrows* have been demonstrated in other tissues, but not yet in ACC. *Red* up-regulation, *green* down-regulation. In other tissues, nuclear *miR-483-5p* is involved in a positive regulatory loop with *IGF-2*, and Wnt/β-catenin signaling stimulates *miR-483* transcription. Cytoplasmic *miR-483-3p* and *miR-483-5p* have been demonstrated to interact with different pathways. Although numerous data support the relevance of *miR-21* and *miR-210* in other tumors, the effects of these microRNAs have not been validated in ACC, yet

MicroRNAs in adrenocortical cancer

Several pathways have been shown to be implicated in ACC pathogenesis including insulin-like growth factor 2 (IGF2) signaling, the tumor suppressor *P53*, *Wnt*/β-catenin pathway etc., but we are far from an overall picture [2, 17]. Several differentially expressed microRNAs have been described among malignant and benign adrenocortical tumors and normal adrenocortical tissue. Here we discuss those significantly differentially expressed ACC-associated microRNAs in detail that have been validated in at least two independent studies (microRNAs highlighted in bold in Table 1).

Relevance of microRNAs in ACC pathogenesis: differentially expressed microRNAs and affected pathways

Among the overexpressed, oncogenic microRNAs in ACC, *miR-483-5p* and *miR-483-3p*, representing the two arms of the miR precursor transcribed from the *miR-483* gene, are among the most consequently overexpressed microRNAs [18–20]. Its pathogenic role in ACC is supported by findings showing that inhibition of both *miR-483-3p* and *miR-483-5p* in vitro resulted in decreased cell proliferation, but only decreased *miR-483-3p* was associated with increased apoptosis [20]. The *miR-483* gene is located within the second intron of the *IGF2* gene, which is one of the most frequently overexpressed genes in ACC [17]. Furthermore, nuclear *miR-483-5p* was shown to enhance *IGF-2* transcription via a hypothesized positive feedback loop in a Ewing sarcoma cell line [21], and if such a mechanism would exist in ACC, this would add a further layer of complexity in *miR-483* action. *miR-483-5p* is activated by the Wnt/β-catenin pathway in lung adenocarcinoma [22], and affects cyclin–dependent kinase expression [23], but these interactions have not been demonstrated in ACC, yet. Overexpressed *miR-483* can thus be hypothesized to be a major factor in ACC pathogenesis (Fig. 1 and Table 2).

Overexpressed *miR-503* has been validated in ACC [20, 24]. The molecular pathways affected by *miR-503* in ACC are unknown, but mRNAs of proteins involved in cell signaling and the cell cycle have been validated in other tumors [25, 26] (Table 2).

miR-210 overexpression has been described in a wide range of different malignancies including ACC [20, 24]. *miR-210* is induced by hypoxia inducible factor 1α (HIF1α) and is the major hypoxia-associated microRNA overexpressed under hypoxic conditions often encountered in malignant neoplasms [27]. *miR-210* is involved in the regulation of angiogenesis, mitochondrial energy metabolism, DNA repair, apoptosis and the cell cycle via affecting a wide range of mRNAs [27]. In a most recent study, *miR-210* overexpression has been associated with aggressive ACC behavior characterized by necrosis and high Ki-67 proliferative index [28].

Overexpressed *miR-675* is interesting [29], as its gene is embedded in the first exon of the long non-coding RNA *H19* [28] forming part of the imprinted *IGF2/H19* locus [17]. The receptor for IGF2, insulin-like growth factor receptor 1 is a potential target of *miR-675* [30]. Since *H19* is usually underexpressed in ACC [31], the overexpression of *miR-675* is difficult to explain.

Table 2 Validated mRNA targets and affected pathways of microRNAs relevant in ACC

microRNA	Over- or underexpressed in ACC	Validated targets in the adrenal	Pathways affected	Reference	Validated targets in other tissues	Pathways affected	Reference(s)
miR-483-3p	Overexpressed	*PUMA*	Apoptosis	[20]	*CDC25A*	Cell cycle	[23]
miR-483-5p	Overexpressed				*RhoGDI1, ALCAM*	Cell migration, invasion growth factor	[24]
					IGF-2		[22]
miR-503	Overexpressed				Phosphatidyl-inositol 3-kinase, inhibitor of κB kinase β, cyclin D1	Signaling	[25]
						Signaling	[26]
						cell cycle	
miR-195	Underexpressed	*DICER, TARBP2*	microRNA processing	[41]	Cyclin D1, cyclin E1, *CDC42,*	Cell cycle	[33]
					Stim 1,	Cell cycle	[34]
					VEGF	Cell migration	[35]
						Angiogenesis	[36]
miR-497	Underexpressed	*DICER, TARBP2*	microRNA processing	[41]	Checkpoint kinase 1, cyclin E1	Cell cycle	[39]
						Cell cycle	[40]
miR-335	Underexpressed				Formin family of actin nucleators	Cell migration, invasion	[44]
					BCL2L2	Apoptosis	[45, 46]
							[47]
*miR-99a**	Underexpressed	*IGFR1, mTOR*	Signaling	[48]	*IGFR1, mTOR*	Signaling	[49]
*miR-100**	Underexpressed	*IGFR1, mTOR*	Signaling	[48]	*PLK1*	Cell cycle	[50]
					FKBP51	Signaling	[49]

ALCAM activated leukocyte cell adhesion molecule, *BCL2L2* B cell CLL/lymphoma 2 like 2, *PUMA* p53 upregulated modulator of apoptosis, *CDC25a* cell division cycle 25a, *CDC42* cell division cycle 42, *PLK1* polo-like kinase 1, *RhoGDI1* rho GDP dissociation inhibitor alpha, *Stim1* stromal interaction molecule 1, *VEGF* vascular endothelial growth factor, *validated in pediatric adrenal tumors

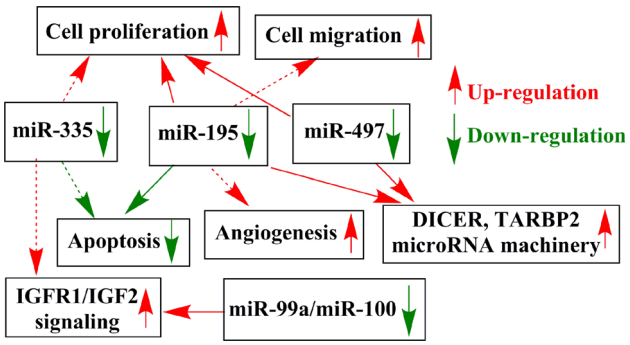

Fig. 2 Schematic representation of the potential relevance of underexpressed microRNAs in ACC pathogenesis. *Continuous arrows* represent validated interactions in ACC, whereas *dashed arrows* have been demonstrated in other tissues, but not yet in ACC. *Red* up-regulation, *green* down-regulation

Several underexpressed microRNAs have been validated in ACC. Down-regulation of the tumor suppressor *let-7* microRNA family member *miR-195* has been described in several studies [18–20, 32] and overexpressed *miR-195* inhibited ACC proliferation in vitro [20]. *miR-195* affects pathways involved in the cell cycle, cell migration, signaling, and angiogenesis [33–36]. Vascular endothelial growth factor (VEGF) is among its validated targets in hepatocellular carcinoma [35], and underexpressed *miR-195* might be involved in the overexpression of *VEGF* characteristic for ACC [37]. By our in silico gene expression network analysis, underexpressed *miR-195* forms part of the core layer of the regulatory network as a major hub interacting with several other genes including the *c-MYC* oncogene [38]. Another *let-7* family member, *miR-497*, has also been found to be underexpressed in ACC, and its overexpression in vitro has lead to decreased cell proliferation in adrenocortical cells [20] and in other tissues it was shown to affect cell cycle regulation [39, 40] (Fig. 2 and Table 2).

It is interesting to note that three microRNA-processing enzymes, i.e., *DICER, TARBP2*, and *DROSHA*, are overexpressed in ACC compared to adrenocortical adenoma (ACA), and among these, *DICER* and *TARBP2* appear to be targets of both *miR-195* and *miR-497* [41]. This observation highlights the remarkable interplay between microRNAs and the microRNA-processing machinery, whereby microRNAs themselves might be implicated in the dysregulation of microRNA expression in ACC.

miR-335 underexpression has been validated in three studies [19, 29, 32] and this microRNA is considered to be a potent tumor suppressor microRNA in several human cancer tissues [42, 43]. No data are available yet on its targets in the adrenal cortex, but in other tissues *miR-335* suppresses tumor cell migration, invasion, and metastasis [44–47] (Table 2).

In the only study involving pediatric adrenal tumors, *miR-99a* and *miR-100* were identified as the most significantly down-regulated microRNAs. *IGFR1* and *mTOR* (mammalian target of rapamycin)/raptor mRNAs were established as targets of these microRNAs in both adrenal and other tissues [48, 49] along with targets involved in cell cycle regulation [50] (Table 2).

From the pool of several other differentially expressed microRNAs described in adrenocortical tumors, but unconfirmed in two independent studies, underexpressed *miR-125b* [18] and overexpressed *miR-21* [20] are worth mentioning. *miR-125b* behaves as a tumor suppressor in several malignancies often accompanying *miR-100* that can be related to their close chromosomal localization (11q) [51]. *miR-21* is a widely overexpressed oncogenic microRNA [52] that affects many pathways involved in the regulation of the cell cycle, receptor signaling, apoptosis, etc. [53–55] (Table 2).

By analyzing the published microRNA profiling studies, we have found 39 microRNAs that are commonly altered in at least two reports [56]. The pathways affected by differentially expressed microRNAs involved the cell cycle, retinoic acid signaling (including aryl hydrocarbon and integrin signaling), and several other pathways whose biological relevance in ACC is unclear [56]. Altered expression of mRNAs coding for proteins regulating the G2-M cell cycle checkpoint has been established as the major microRNA-mediated pathway in our microRNA profiling study by bioinformatics tools [24]. In line with these data, cell-cycle checkpoint regulation and retinoic acid signaling have been identified as major pathogenic pathways in ACC by our meta-analysis of transcriptome datasets [57].

In a most recent study, next-generation sequencing (NGS) has been performed in a cohort of 45 ACC samples [58]. By the analysis of microRNA expression, three major tumor clusters could be established that could be correlated with the clinical behavior of ACC along with the other molecular alterations. Apart from overexpressed *miR-483-5p*, novel microRNAs including the up-regulated *miR-506-514* cluster have been found [58]. This is the only study using the NGS approach to date for microRNA profiling in ACC, and the validation of the significantly differentially expressed microRNAs should be performed by another technique (like real-time PCR). Nevertheless, the molecular subclassification of ACC based on bioinformatics approaches is a major finding from a clinical point of view, as it could be used to select patients with the risk of recurrence and rapid progression for whom aggressive treatment strategy is warranted.

Despite several commonly altered microRNAs in different studies, an overall picture of microRNA expression changes in ACC is lacking. In order to obtain more

homogeneous and conclusive data, international collaborative studies would be helpful to enable profiling on large sample sets on uniform methodological platforms (e.g., microarray or NGS validated by PCR-based methods) using uniform statistical analysis.

MicroRNAs in the diagnosis of adrenocortical cancer

Intensive research efforts are being conducted to find adrenocortical microRNA markers of malignancy and prognosis. The histological diagnosis of adrenocortical tumors is difficult, and the mostly used Weiss-scoring has severe limitations [2]. It is very difficult to exclude malignancy in large (>6 cm diameter) tumors and to establish malignancy in small (<4 cm diameter) tumors.

Overexpressed *miR-483-5p* [18, 19], underexpressed *miR-195* [19], the expressional difference of *miR-503* and *miR-511* [24], and overexpressed *miR-335* and *miR-675* [29] have been proposed as reliable markers of malignancy. Overexpressed *miR-483-5p* [19], *miR-503*, *miR-1202*, and *miR-1275* [20] and underexpressed *miR-195* [19] have been associated with poor survival in ACC. Despite the high sensitivity and specificity values reported for some of these microRNAs (e.g., 100 % positive predictive and 92 % negative predictive value for *miR-483-5p* [18] and 100 % sensitivity and 97 % specificity for *miR*-511-*miR-503* [24]), the patient cohorts were mostly small and therefore larger-scale studies are warranted to confirm the clinical utility of these tissue microRNAs. Moreover, the hormonal features of tumors have been taken into account in two studies [24, 29], whereas others have included samples irrespective of their hormonal activity [18–20]. Since microRNA patterns can be associated with differences in hormone secretion [59], such discrepancies in the composition of patient cohorts might be relevant [60]. For the better evaluation of the diagnostic or prognostic role of certain microRNAs in adrenocortical tumors, the direct assessment of microRNAs in adrenal tissues by in situ hybridization would be a novel possibility [61].

Circulating microRNAs might be exploited as minimally invasive markers of malignancy enabling an early preoperative diagnosis [9]. Three studies have been published to date reporting on the analysis of circulating microRNAs in adrenocortical tumors. Overexpression of *miR-483-5p* paralleling observations made in ACC tissues has been confirmed in all studies [31, 62, 63]. In the study by Chabre et al., overexpressed *miR-483-5p* and underexpressed *miR-195* could be exploited for the differentiation for aggressive and non-aggressive ACC [31] that could be of major relevance for treatment planning. Overexpression of *miR-100*, *miR-181b*, *miR-184*, and *miR-210* in ACC plasma samples have been noted in our study [62], whereas overexpressed *miR-34a* has been described in Patel's study [63]. The overexpression of serum *miR-34a* is difficult to explain as it is mostly regarded as a tumor suppressor and is underexpressed in several malignancies [64] including ACC by microarray [18]. The sensitivity and specificity values reported for these blood-borne microRNAs do not appear to be high enough for clinical introduction as malignancy markers at present [62, 63].

There are several technical difficulties associated with the analysis of circulating microRNAs [62, 65, 66]. The low RNA yield of blood samples and the lack of reliable methods for quantity assessment of blood-borne microRNAs makes the standardization of data difficult. The profiling method is also a matter of debate, since microarray, PCR, and most recently NGS have been proposed. We suggest the use of PCR for circulating microRNA profiling [62], but NGS might also be promising in the future. The method of raw data normalization, the choice of reference gene is another major issue. Synthetic spike-in-RNAs (e.g., *cel-miR-39*) has been used as reference in several studies including ours [62], but these cannot be regarded as biological controls. Circulating microRNAs with relatively stable expression like *miR-16* was useful in some studies [62, 65], and the combination of different reference genes can also be proposed [62].

MicroRNAs in pheochromocytomas

MicroRNA expression profiling in pheochromocytomas (PCC) and paragangliomas (PGL, extraadrenal pheochromocytoma) has been reported in four studies to date. It was found that 25–30 % of PCC arise in the context of hereditary tumor syndromes including multiple endocrine neoplasia type 2 (MEN2), von Hippel-Lindau disease (VHL), neurofibromatosis type 1 (NF1), hereditary paraganglioma syndromes caused by germline mutations of *RET*, *VHL*, *NF1*, *SDHD*, *SDHAF2*, *SDHC*, *SDHB* and *SDHA* (SDH: succinate dehydrogenase) genes, respectively [67]. Recently, mutations of two novel genes, *TMEM127* and *MAX* have been found to be associated with PCC as well [68, 69]. The molecular pathogenesis of PCC follows two main routes: (i) pseudohypoxia-neoangiogenesis pathways linked to the overactivation *HIF1α* (mutations in *SDH* and *VHL* genes, cluster 1), (ii) activation of PI3K/AKT/mTOR/Ras signaling characteristic for *RET*, *NF1*, *TMEM127*, and *MAX* mutations (cluster 2) [67]. These two clusters are also clearly distinct in their transcriptional (mRNA) profiles [69]. PCCs are mostly benign, but their benign or malignant behavior cannot be predicted by histological analysis [3, 4]. Malignancy is frequent in SDHB-associated PCC. There are no reliable markers of malignancy or recurrence [3, 4], and microRNAs might be promising in this respect.

Table 3 Validated microRNAs significantly differentially expressed in pheochromocytomas reported in four studies

	General PCC	MEN2	VHL	SDHB	SDHD	MAX	Recurring PCC	Malignant PCC
de Cubas et al. [71]	miR-137↑*	miR-382↑	miR-382↑	miR-382↑	miR-382↑	miR-382↓		
		miR-488↑	miR-210↑	miR-210↑				
		miR-885-5p↑	miR-133b↑	**miR-183↑**				
				miR-96↑				
Patterson et al. [73]				miR-483-5p↑				**miR-483-5p↑****
				miR-101↑				miR-101↑
				miR-183↑				**miR-183↑**
Tömböl et al. [72]		**miR-885-5p↑**	miR-139-3p↑				miR-1225-3p↑	
			miR-541↑					
			miR-765↑					
Meyer-Rochow et al. [87]								**miR-483-5p↑**
								miR-15a↓
								miR-16↓

microRNAs in *bold* have been described in two studies

↓ down-regulation, ↑ up-regulation relative to sporadic PCC, * except for MAX-related PCC, ** Patterson et al. studied only SDHB-associated malignant PCC

Table 4 Validated mRNA targets of microRNAs relevant in pheochromocytoma

microRNA	Validated targets in the adrenal	Principal function of target mRNA	Reference	Validated targets in other tissues	Principal function of target mRNA	Reference
miR-96	Ezrin	Cell adhesion, migration	[70]	*FOXO1*	Transcription factor	[75, 76]
miR-183	Ezrin	Cell adhesion, migration	[70]	SMAD4	Signaling, apoptosis	[77]
				Isocitrate dehydrogenase 2	Energy metabolism	[78]
miR-885-5p				Caspase 3,	Apoptosis	[80]
				CDK2, MCM5	Cell cycle	[81]
miR-137				*KDM1A*,	Gene expression regulation, Cell signaling	[82]
				Ezh2 histone methyltransferase, neurofibromin 1		[83]
						[84]
miR-382				*c-MYC*	Cell cycle, apoptosis	[85]
miR-15a				*BCL2, MCL1*	Apoptosis	[88]
				Cyclin D1	Cell cycle	
miR-16				*BLC2, MCL1*	Apoptosis	[88]
				Cyclin D1	Cell cycle	

BCL2 B-cell CLL/lymphoma 2, *CDK2* cyclin-dependent kinase 2, *KDM1A* histone demethylase, lysine-specific demethylase 1, *MCL1* myeloid cell leukemia 1, *MCM5* mini-chromosome maintenance complex component 5

MicroRNA profiling has been performed in hereditary tumor syndrome-associated PCC (Table 3). MicroRNAs as genetic group markers have been established characteristic for clusters 1 and 2 similar to the clustering based on the transcriptome. Overexpression of hypoxia-related *miR-210* was found in pseudohypoxia-associated highly vascularized *SDHB*- and *VHL*-associated tumors [71]. Overexpression of *miR-139-3p*, *miR-541*, *miR-765*, and *miR-133b* have been found characteristic for VHL tumors [71, 72]. Overexpressed *miR-96* [71] and *miR-183* [71, 73] was described in *SDHB*-associated pheochromocytomas, and the transfection of these two microRNAs hampered the nerve growth factor (NGF)-induced differentiation of rat PC12 cells in vitro [70]. NGF treatment of PC12 resulted in the significant underexpression of *miR-139-3p* and *miR-210*, which are overexpressed in *VHL*- and *SDHB* + *VHL*-

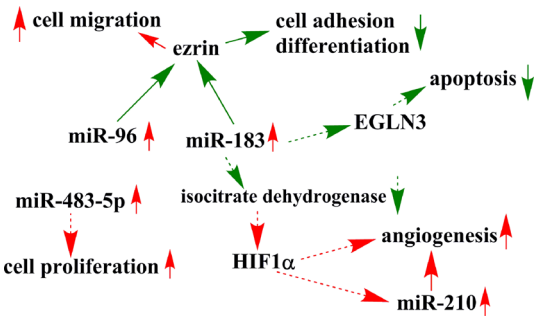

Fig. 3 Schematic representation of the potential relevance of microRNAs in SDHB-mutation-associated pheochromocytoma. *Continuous arrows* represent validated interactions in PCC, whereas *dashed arrows* have been demonstrated in other tissues, but not yet in PCC. The interaction between *miR-183* and EGLN3 has been hypothesized in PCC, but it has not yet been validated. *Red* up-regulation, *green* down-regulation

tumors, respectively [74]. Furthermore, the cytoskeleton-associated protein ezrin, involved in cell adhesion, migration, and neuronal differentiation, was validated as a target of *miR-96* and *miR-183* [71]. *miR-96* and *miR-183* belong to the "miR-183 family" of oncogenic microRNAs that are overexpressed in a wide array of human cancers [75–77] (Table 4). Overexpressed *miR-183* was hypothesized to interfere with *EGLN3* (EGL-nine) expression involved in the regulation of neuronal apoptosis [71]. Moreover, *miR-183* has been shown to up-regulate *HIF1α* via targeting isocitrate dehydrogenase 2 in glioma [78]. If a similar interaction could be demonstrated in SDHB-associated PCC, this might be implicated in the pseudohypoxia phenotype (Fig. 3). In our meta-analysis on neural crest tumors (PCC and neuroblastoma), we have noted the overexpression of *EGLN* mRNA in PCC that could be correlated with down-regulated *miR-132* in VHL-associated tumors [79].

Overexpression of *miR-885-5p* was observed in MEN2-associated PCC [71, 72] together with *miR-488* [71]. *miR-885-5p* appears to target mRNAs of proteins involved in the regulation of apoptosis [80] and cell cycle [81] in other tissues (Table 4). By our in silico meta-analysis, the involvement of insulin-like growth factor 1 (IGF1) pathway in the pathogenesis of MEN2-associated PCC has been raised, and *miR-885-5p* might target IGF-binding proteins (*IGFBP3, IGFBP7*) [78].

Two microRNAs, *miR-137* and *miR-382*, were overexpressed in most PCC, and therefore can be regarded as general PCC markers [72]. In *MAX*-mutation-related PCC, however, these two microRNAs were underexpressed, thus MAX-mutations might result in a microRNA profile distinct from other PCC classes. *miR-137* is underexpressed in several malignancies and its targets include mRNAs involved in gene expression regulation and signaling [82–84]. The oncogene *c-MYC* was found among the validated targets of *miR-382* [85] that might be relevant as *MAX* (MYC-associated factor X) forms part of the *c-MYC* network [69].

We have observed the overexpression of *miR-1225-3p* in recurring PCC compared to sporadic non-recurring, MEN2-, VHL-, and NF1-associated PCC. By pathway analysis, overexpressed *miR-1225-3p* might result in decreased Notch signaling [72]. The relevance of Notch signaling is underlined by in vitro studies on rat PC12 PCC cells where histone deacetylase inhibitors up-regulating Notch-1 inhibited cell proliferation [86]. Notch signaling might thus represent a novel target of PCC treatment.

The microRNA expression in malignant PCC has been investigated in two studies [73, 87] (Table 3). Overexpressed *miR-483-5p* was described in both studies. *miR-483-5p* is thus overexpressed in both malignant adrenocortical and adrenomedullary tumors despite their different embryogenic origin (cortex is mesodermal, medulla is ectodermal). Underexpressed *miR-15a* and *miR-16* described by Meyer-Rochow et al. in malignant PCC [87] can be regarded as general tumor suppressor microRNAs down-regulated in several neoplasms targeting mRNAs of proteins involved in apoptosis and cell cycle regulation [88]. Transfection of *miR-15a* and *miR-16* to rat PC12 cells inhibited their growth supporting their tumor suppressing activity in PCC, as well [87]. Three circulating microRNAs (*miR-101, miR-183*, and *miR-483-5p*) have been investigated as markers of PCC malignancy, but no significant differences in expression were found [73].

Perspective for microRNAs as direct or indirect targets for treatment

Being involved in basic cell biological processes leading to cancer development, microRNAs can be regarded as potential and potent targets for anti-cancer therapy. MicroRNAs could represent both direct and indirect targets. Here we mean direct targeting if the microRNA is directly affected by molecular approaches (antagonizing oncogenic miR by e.g., antagomirs, or increasing endogenous tumor suppressor microRNA expression by microRNA mimics, etc.) [14], whereas by indirect targeting the pathways established by microRNA profiling studies could be affected.

MicroRNAs as direct targets

Given their tissue-specific way of action, selecting microRNAs as treatment targets is difficult. Those microRNAs could be preferred, the expression of which is changed in the same direction in a wide array of malignancies. MicroRNAs have been proposed to overcome tumor chemo- and radiation resistance [5, 89]. We would propose those microRNAs for consideration whose targets

and affected pathways have already been described and validated in several different tumor models. From the pool of relevant microRNAs in ACC and validated in at least two independent studies, *miR-483-5p/miR-483-3p*, *miR-195*, and *miR-210* would be the most suitable candidates.

There are some examples on the potential anti-tumor applicability of these microRNAs in other tumors. In breast cancer cells, up-regulation of *miR-195* expression increased their sensitivity to adriamycin treatment [90]. Knockdown of *miR-210* facilitated radiation therapy in a human hepatoma xenograft model [91].

There are several major difficulties associated to the modulation of endogenous microRNA expression. Since the major goal of microRNA modulation would be most valuable in advanced tumor patients, systemic therapy would be envisaged. Apart from technical issues such as problems of administration, major unforeseen side effects could develop in part due to potential off-target actions of microRNAs related to the tissue-specific nature of microRNA action [14].

Indirect targets: pathways affected by microRNAs

The pathways established by microRNA profiling studies could also serve as therapeutical targets. The mTOR/Raptor signaling affected by *miR99a/miR100* in childhood ACC could be influenced by the mTOR inhibitor everolimus demonstrated by in vitro and xenograft observations [48]. Despite these promising experimental data, however, everolimus does not appear to be an efficient therapeutic modality for ACC in the clinical setting [92]. Notch-signaling might be another example established in our study on recurring PCC [72] that could be exploited in the treatment by e.g., histone deacetylase inhibitors [86].

Among the other potential pathways that can be suggested by microRNA studies, retinoic acid signaling in ACC [56] might be of interest, and our in vitro and xenograft studies support the potential applicability of 9-*cis*-retinoic acid in ACC treatment [93]. Other, but currently unvalidated pathways such as aryl hydrocarbon and integrin signaling [56] might also include targets that could be exploited in therapy.

Studies on gene expression networks might be helpful for identifying the most relevant targets [38].

Since both ACC and malignant PCC are rare tumors, rapid advances in the clinical introduction of microRNA modulating approaches are hardly expected.

Conclusions

Altered expression of microRNAs has been described in several studies both in adrenocortical and adrenomedullary tumors. Their implication in adrenal tumorigenesis appears to be relevant, and their diagnostic utility is promising both as tissue and circulating biomarkers. There are, however, major discrepancies in the results from different research groups that can be associated with the different platforms, methodological approaches, patient cohorts, etc. Extensive experimental work-up and validation on larger patient cohorts will be needed to define the microRNA sets that can be reliably used for diagnosis. There is great potential for microRNAs in cancer treatment, but the clinical applicability of direct microRNA targeting for adrenal tumors seems to be, however, quite far away.

Acknowledgments This study has been supported by the Hungarian Scientific Research Fund (OTKA K100295).

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

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