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Circulating MicroRNAs in Cutaneous Melanoma Diagnosis and Prognosis

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Published Date: April 10, 2016

ABSTRACT

Cutaneous melanoma represents a challenge for pharmacologists and clinicians due to their high degree of genetic and phenotypic heterogeneity. The identification of new non-invasive and informative biomarkers would therefore represent a substantial step to adequately treat melanoma patients. MicroRNAs (miRNAs) are a class of small non-coding RNAs that play an important role as negative regulators of gene expression. Several studies have demonstrated their correlation with disease status in different types of cancer including melanoma. Extracellular miRNAs are released from both tumor cells and/or normal cells. MiRNAs do not circulate freely in biological fluids but they are incorporated into extracellular vesicles or form complexes with lipids and proteins. Circulating miRNAs may represent potential biomarkers of cutaneous melanoma diagnosis and patient prognosis. Longitudinal monitoring of cell-free miRNAs in biological fluids of melanoma patients could help clinicians to predict disease progression before the tumor becomes resistant to a given drug. However, to confirm their clinical utility it will be necessary to validate the best available technique for their detection and quantification and to test selected miRNAs in prospective clinical trials.

Keywords: Cutaneous melanoma; Circulating microRNA; Exosomes; Diagnostic biomarker

MICRORNA GENERALITY

In the mammalian genome, the known protein-coding transcripts derives from only about 2% of total DNA, but at least 60-70% of total DNA is transcribed in RNA. Approximately 20 years ago, researchers determined that components of the genome traditionally considered non-functional had, instead, gene regulatory capacity [1]. Indeed, multiple types of non-coding RNAs, both long and small, have evolved in eukaryotes to regulate genetic materials and transcripts [2,3].

MicroRNAs (miRNAs) are a conserved class of small non-coding RNAs that function as negative regulators of gene expression [4]. MiRNA biogenesis (Figure 1) begins with transcription of a long precursor (pri-microRNA, several hundred nucleotides [nts]) by RNA polymerase II in the cell nucleus. These pri-miRNAs with a stem-loop structure are firstly recognized by the nuclear ribonuclease DROSHA (a RNase III family member) and by double-stranded RNA-binding proteins partners (e.g., DGCR8), and then cropped into a shorter second miRNA precursor, pre-miRNA (70 nts stem-loop). These precursors are transported into the cytoplasm by Exportin-5/Ran-GTP and then cleaved into a double-stranded miRNA molecule of driver and passenger strands, the miRNA-miRNA* duplex (21 nts each strand), through a second processing step carried out by the ribonuclease DICER (an endoribonuclease of the RNase III family) and by the transactivation-responsive RNA-binding protein (TRBP). Both strands can act as mature miRNAs, but generally only one driver (guide) strand is incorporated into a miRNA-induced silencing complex (miRISC) becoming a functional miRNA, while the passenger strand (miRNA*) is quickly degraded most of the times [5,6].

miRISC complex binds mainly to the 3' untranslated regions (UTRs) of specific target mRNAs leading to the repression of protein expression and promotion of target mRNA degradation. It has been recently reported that miRNAs can also bind to ribonucleoproteins in RISC-independent manner and interfere with their RNA binding functions (decoy activity) [7]. Three studies have demonstrated that miRNAs can also regulate gene expression at the transcriptional level by direct DNA binding [8-10].

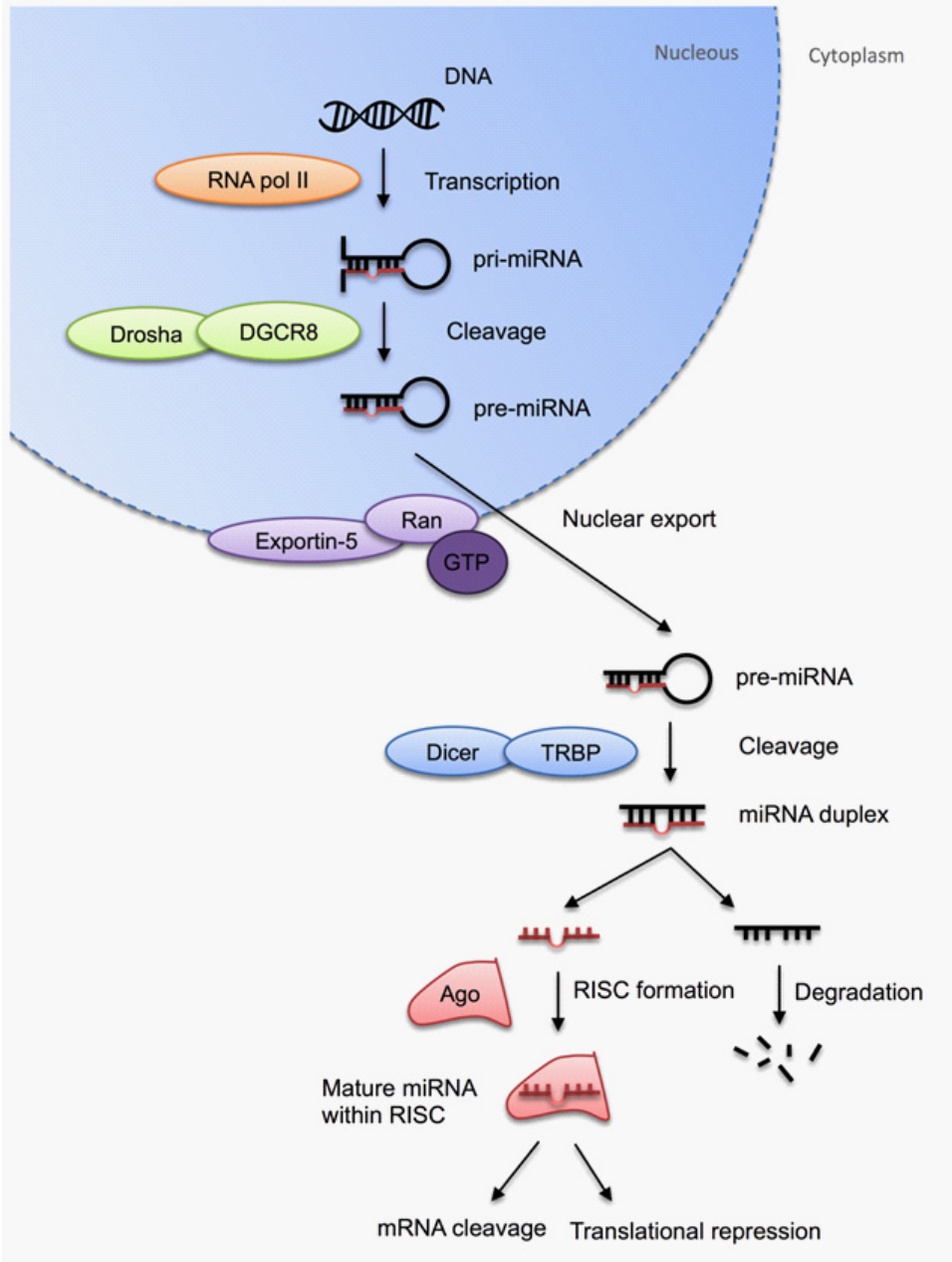

Figure 1: The canonical pathway of miRNA biogenesis. MiRNA genes are transcribed by RNA polymerase II forming pri-miRNAs in the cell nucleus. Pri-miRNAs are cleaved by Drosha/DGCR8 (forming a shorter second pre-miRNA), transported into the cytoplasm by Exportin-5/Ran-GTP, and then processed by Dicer/TRBP to produce miRNA duplex. One strand of miRNA duplex is incorporated into miRNA-RISC complex leading to the repression of protein expression and promotion of target mRNA degradation.

Currently, over 2000 human miRNAs have been identified in the human genome; however, the real number is expected to be much higher [11]. Each miRNA may have the potential to repress the expression of hundreds of target genes, highlighting the important role of this specific form of regulation in cell identity and disease, in mammals [12-15].

As a matter of fact, gene regulation by miRNAs has been linked to proliferation, differentiation, survival, stress response and apoptosis, which represent some of the biological events primarily altered during the pathophysiological processes that characterized several human diseases, including cancer [6].

Up to a few decades ago, it was believed that tumorigenesis was almost exclusively caused by alterations in oncogenes and/or tumor suppressor genes [16]. The discovery of small non-coding RNAs has shed light on the complex mechanisms involved in cancer development and progression. Noteworthy, miRNAs' expression profiles in healthy cells are markedly deregulated in solid and hematopoietic tumours, where they seems to have tumour suppressive or tumour promoting activities, depending on the nature of their specific target. Therefore, differential miRNA expression analysis may be important to identify novel biomarkers that might help clinicians to improve diagnosis and treatment of patients. For example, specific miRNA profiles have been associated to tumour development, progression, prognosis and response to treatment, suggesting the existence of tumor-specific miRNA fingerprints [17]. The causes of the abnormal expression of genes coding for miRNA in malignant *versus* normal cells could be explained taking into account the location of these genes in cancer-associated genomic regions, epigenetic mechanisms and/or alterations in the miRNA processing machinery [16,18]. Actually, the fact that different types of cancer can be discriminated by miRNA profiling [18] points at miRNA as potential diagnostic and prognostic indicators of disease type and severity.

CIRCULATING miRNAS

Circulating miRNAs have been identified for the first time in 2008 [19] in plasma and serum samples, and subsequently in other body fluids including saliva, breast milk, cerebrospinal fluid and others [20,21].

Circulating miRNAs (Figure 2) are packaged in microparticles (exosomes, microvesicles, apoptotic bodies), circulating tumor cells (CTC), or compexed with either RNA-binding proteins (Argonaute, Ago) or lipoprotein (high-density lipoprotein, HDL). These complexes prevent their degradation making miRNAs stable and protecting them from RNase activity. Recently, circulating miRNAs have been identified as possible mediators of cell-to-cell communication [22].

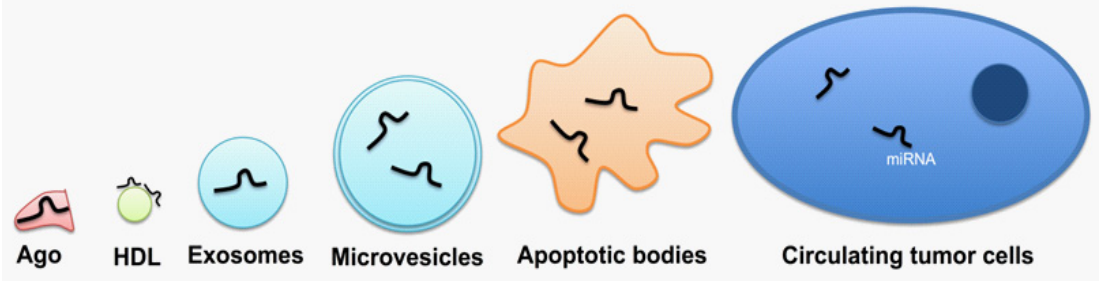

Figure 2: Storage sites of circulating miRNAs (see text for further details).

The level of miRNAs in blood and other body fluids (urine, saliva, sweat) changes as a consequence of altered pathophysiological mechanisms and tissue injury [21].

Mitchell and co-workers [19] showed that tumor cells release miRNAs into the circulation and that serum levels of a tumor-expressed miRNA can select patients with cancer from healthy subjects with significant specificity and sensitivity. Moreover, it has been also suggested that miRNAs might derive from blood cells and/or endothelial cells [23].

Plasma stability, easy detectability, and tumor specificity of circulating miRNAs make them ideal candidates as biomarkers for diagnosis and/or prognosis of a variety of cancer, including melanoma [24].

Circulating miRNAs in Cutaneous Melanoma

Melanoma is the most aggressive skin cancer that needs to be treated as early as possible to obtain the maximum benefit in terms of patient survival. The 5-year survival rate for localized melanoma is 98%; however, this drops to 16% in cases where cancer has metastasized to distant sites or organs [25].

Although current clinical, morphologic, and histopathologic evaluations, melanoma is still an unpredictable disease. In this context, the ability to identify non-invasive biomarkers for disease detection and monitoring could change the prospective of melanoma patients. To date, there are no biomarkers that are sensitive or specific enough to be beneficial for early detection and surveillance of melanoma. Several serologic markers of different biochemical nature have been intensively investigated in the last few years to create a blood test to introduce in the clinical practice. As a matter of fact, the non-specific enzyme lactate-dehydrogenase (LDH) and the BRAF V600E polymorphism are the only molecular determinants used as prognostic and predictive biomarkers in melanoma patients, respectively [26].

Most recently, miRNAs have been studied for their utility as biomarkers in a wide range of malignancies [27,28] and, as far as melanoma is concerned, a number of miRNAs with potentialities as diagnostic or prognostic biomarkers have emerged.

The analysis of the scientific literature carried out on PubMed database using "circulating/blood/plasma/serum miRNA cutaneous melanoma" as key search terms provided a total of 303 miRNAs identified as circulating in melanoma patients (for the complete list please refer to supplementary material). However, only some of them have obtained diagnostic or prognostic significance. They are reported with relative deregulation status in melanoma vs healthy subjects, source (plasma, serum or exosomes) and technique used for their evaluation, in Table 1 and discussed in the following paragraphs.

Table 1: Circulating miRNAs as biomarkers in cutaneous melanoma.

	microRNA		Deregulation	Source	Technique
Diagnosis	miR-186, let-7d-3p, miR-145, miR-99a, miR-664, miR-591-5p, miR-378*, miR-29c-5p, miR-1280, miR-365, miR-1249, miR-328, miR-422a, miR-30d, miR-17-3p, miR-18a-3p [29]		--	Plasma	Microarray (miR-17* and mir-18a* also qRT-PCR)
	miR-10b, miR-145, miR-155 [30]		Down	Plasma	qRT-PCR
	miR-221 [32]		Up	Serum	qRT-PCR
	miR-29c-5p, miR-324-3p [33]		Down	Serum	qRT-PCR
	miR-9, miR-145, miR-150, miR-155, miR-205 [34]		--	Serum	Real-time PCR
	miR-1246, miR-185 [35]		Up	Plasma	Microarray qRT-PCR
	miR-21-5p, miR-320a [36]		Up	Plasma/Serum	Microarray NGS ddPCR
Staging	miR-221 [37]		Up (III-IV stages)	Serum	qRT-PCR
	MELmiR-7	miR-16, miR-211-5p	Up (IV stage)	Serum	qRT-PCR
		miR-4487, miR-4706, miR-4731, miR-509-3p, and miR-509-5p [22]	Down		
	miR-200c-3p, miR-204-5p, miR-182-5p, miR-301a-3p [38]		Down (IV stage)	Serum	qPCR array
	miR-211-5p, miR-193b-3p, miR-720, miR-205-5p [38]		Up	Serum	qPCR array
	miR-211-5p, miR-21 [40]		--	Serum	qRT-PCR
	miR-125b [41]		Down (late stages)	Serum/Exosomes	Real time PCR
Recurrence and Prognosis	miR-150, miR-15b, miR-199a-5p, miR-33a, and miR- 424 [44]		Up	Serum	qRT-PCR

Circulating miRNAs As Diagnostic Biomarker in Cutaneous Melanoma

Several studies have demonstrated the ability of serum or plasma miRNAs to distinguish melanoma patients from healthy subjects. Nonetheless, the specificity of deregulated miRNAs for melanoma as compared to other cancer types has been investigated only in few cases.

In 2010, Leidinger and colleagues [29], after screening almost 900 human miRNAs in blood samples from 20 healthy individuals and 24 melanoma patients, identified 51 deregulated miRNAs (30 up-regulated and 21 down-regulated). Afterwards, they developed a signature of 16 miRNAs (i.e., miR-186, let-7d-3p, miR-18a-3p, miR-145, miR-99a, miR-664, miR-501-5p, miR-378*, miR-29c-5p, miR-1280, miR-365, miR-1249, miR-328, miR-422a, miR-30 d, and miR-17-3p) able to separate melanoma from healthy control samples with high accuracy, specificity and sensitivity.

In the same year, Heneghan et al. [30] analysed whole blood collected from preoperative cancer patients (breast, n=83; prostate, n=30; colon, n=20; and renal cancer, n=20; and melanoma, n=10) and healthy controls (n=63) and found that individual cancers displayed specific systemic miRNA profiles. However, they did not find a miRNA or a panel of miRNAs able to discriminate melanoma disease stages but only some circulating miRNAs dysregulated. Specifically, miR-10b, miR-145 and miR-155 were reduced in plasma samples derived from melanoma patients compared to healthy controls. Furthermore, miR-10b was also found to be significantly lower in blood samples from colon and renal cancer patients, while it was in the normal concentration range in patients with breast and prostate cancers. The level of miR-145 was significantly lower in blood samples from colon and prostate cancer patients, while it was in the normal range for breast cancer. The level of miR-155 was significantly lower in patients with all malignancies except breast cancer.

MiR-221, a miRNA already observed to be up-regulated in melanoma tissues and correlated with malignant phenotype [31], has been evaluated alone in serum samples from 94 melanoma patients and 20 controls. It has been pointed out that miR-221 might be useful for melanoma diagnosis [32]. Nevertheless, the authors observed a significant miR-221 up-regulation in melanoma stage I-IV but not in melanoma stage 0 compared to healthy controls. These findings suggest that miR-221 may not be ideal for early diagnosis.

In 2013, Greenberg and colleagues [33] analysed sera samples obtained from healthy controls (n=20) and patients whit melanoma stage IV (n=28), renal cancer (n=23) and colon cancer (n=20). This pilot study showed that the loss of serum miR-29c-5p and miR-324-3p is highly indicative for metastatic melanoma ($p < 1\times10^{-7}$). miR-29c clearly discriminated melanoma from renal and colon cancer patients, but it could not discriminate between renal and colon cancer. miR-324-3p distinguished melanoma from renal but not colon cancer patients. Moreover, miR-324-3p did not distinguish between renal and colon cancer patients.

Six miRNAs (miR-9, miR-145, miR-150, miR-155, miR-203, and miR-205) were evaluated in the serum of 11 metastatic melanoma patients and 16 patients without melanoma [34]. MiR-9, miR-203 and miR-205 plasma levels were significantly correlated with metastatic melanoma; in particular, combination of miR-9, miR-145, miR-150, miR-155 and miR-205 was more predictive than each miRNA alone.

Other lines of evidence have been produced very recently. For example, a screening analysis of plasma samples from 14 metastatic melanoma patients and 5 healthy subjects identified a

combination of two miRNAs, miR-1246 and miR-185, which were significantly associated with metastatic melanoma [35]. Furthermore, a large clinical investigation provided data suggesting a diagnostic role for miRNAs in melanoma and other cancer types [36]. In this study, the authors analysed plasma and serum samples from healthy controls (n=18) and patients with melanoma (n=8), breast (n=18), colorectal (n=18), lung (n=18), and thyroid (n=27) cancer. They found that only miR-21-5p was consistently increased in the plasma of cancer patients, whereas miRNA-320a appeared to be more cancer specific for melanoma. They also observed that the absolute levels of the same miRNA in serum were different than those in plasma, and they hypothesized that this difference could be linked to the permanence of microvescicle and exosomes in plasma.

Circulating miRNA in Cutaneous Melanoma Staging

Kanemaru et al. [37] analysed serum samples from 94 melanoma patients and 20 healthy controls and found that miR-221 levels did not discriminate among stage I and IV melanomas but resulted increased in stages I-IV compared to healthy subjects or patients with *in situ* melanoma. Moreover, miRNA-221 serum levels decreased after surgical removal of the primary tumor and it increased again after disease recurrence.

The same miRNA was investigated also by Li et al [37], who assessed serum levels of expression of miR-221 in 72 stage I-IV patients and in 54 healthy controls and found that miR-221 increased expression was statistically significant only in stages III-IV patients and correlated with melanoma thickness, poor differentiation, T and N classification and presence of metastasis. This suggests that miR-221 overexpression might be involved in melanoma development and progression. In addition, miR-221 overexpression may act as a negative prognostic factor in melanoma patients.

The levels of a panel of seven circulating miRNAs (i.e, MELmiR-7: miR-16, miR-211-5p, miR-4487, miR-4706, miR-4731, miR-509-3p, and miR-509-5p) were shown to be different between melanoma patients and healthy subjects with high sensitivity (93%) and specificity (≥82%) in the recent study of Stark et al. [23]. This panel was reported to be superior to LDH and S100B markers in predicting melanoma progression, recurrence, and survival.

A profound deregulation in circulating miRNAs was found by Margue et al. [38] investigating the whole miRNome in 30 healthy subjects and 52 melanoma patients They reported, in fact, that in late stage melanoma patients, several miRNAs were profoundly deregulated: miR-200c-3p, miR-204-5p, miR-182-5p, and miR-301a-3p were down-regulated and miR-211-5p, miR-193b-3p, miR-720, and miR-205-5p were up-regulated. In particular, miR-182-5p plasma levels were lower in stage III patients than in healthy volunteers, whereas miR-211-5p levels were 68-fold higher in serum samples from stage IV melanoma patients compared to healthy controls. Since miR-211-5p is a marker for melanocytic cell lineage [39]. Margue et al. [38] hypostasized that it could be secreted also by melanoma cells *in vivo*. Others confirmed these findings [40] by showing that miR-211-5p is overexpressed in plasma of advanced melanoma patients and miR-211 overexpression correlates with melanoma burden.

Alegre et al. [41] compared the levels of miR-125 in serum and in circulating exosome from 21 advanced melanoma patients and 19 healthy volunteers. They found that miR-125b is down-regulated in melanoma patients. Morever, exosomes were found to be a more accurate source of miRNA than serum for measuring circulating miRNA [42].

Circulating miRNA in Predicting Melanoma Recurrence And Prognosis

Only two studies were found in literature suggesting a direct prognostic value of miRNAs in cutaneous melanoma. Ono et al. [43] analysed plasma samples from 218 melanoma patients (stage III and IV) and 35 healthy controls and found that levels of circulating miR-210 have been associated with systemic recurrence prior to its clinical evidence. These data suggest that miR-210 might be able to anticipate recurrence and outcome in advanced melanoma patients.

Friedman et al. [44] screened 355 miRNAs in sera derived from 80 melanoma patients and found detectable expression of 170 miRNAs, five of which (i.e., miR-150, miR-15b, miR-199a-5p, miR-33a, and miR- 424) were significantly associated with recurrence-free survival. This miRNA panel allowed to classify patients with high and low recurrence risk disease.

FUTURE PERSPECTIVE

Circulating miRNA analysis seems to have promising clinical utility as markers of diagnosis, staging, disease recurrence and prognosis in cutaneous melanoma. Nevertheless, before they can be fully utilized in the clinical practice several issues need to be addressed: lack of reproducibility because of absence of universally accepted endogenous control for normalization, a wide variety of evaluation techniques, individuation of cancer specificity and finally validation on prospective clinical trials. Although the studies reported from cutaneous melanoma patients are actually too few and they need to be furtherly validated in large prospectic trials, they have opened a real way forward to get the application of miRNAs in supporting the diagnosis and therapy of cutaneous melanoma.

A field largely unexplored is that about the role of circulating miRNA in predicting drug responsivenes and/or toxicity, that may be of great relevance to drive towards a more personalized therapy.

ACKNOWLEDGEMENTS

Polini B. was supported by Associazione contro il Melanoma (ACM onlus); Carpi S. was supported by a Grant from SIF (Italian Pharmacological Society)-MSD Foundation.

Supplementary S1: Circulating miRNA in melanoma patients

The 303 miRNA circulating comprising: miR-9, miR-9-5p, miR-10b, miR-15b, miR-16, miR-16-1-3p, miR-16-2-3p, miR-17-5p, miR-18b-3p, miR-20a, miR-20a-3p,miR-21-3p miR-21-5p, miR-22-3p, miR-23a-5p, miR-29c, miR-30d, miR-33a, miR-33a-5p, miR-34a, miR33b-5p, miR-34C3P, miR-92b-5p, miR-96-5p, miR-98-5p, miR-99a-3p, miR-99a-5p, miR-99B-3p, miR-101-5p,

miR-103, miR-103a-3p, miR-106a, miR-122-5p, miR-124-3p, miR-125a-5p, miR-125b, miR-125b-2-3p, miR-127-5p, miR-129-5p, miR-132-5p, miR-133a-3p, miR-133b, miR-135a-5p, miR-135b-3p, miR-135b-5p, miR-136-5p, miR-138-5p, miR-142-5p, miR-143-5p, miR-145-5p, miR-146a-5p, miR-146b-3p, miR-147b, miR-148a-5p, miR-148b-3p, miR-149-5p, miR-150, miR-155-3p*, miR-155-5p, miR-181a-5p, miR-185, miR-185-3p, miR-187-3p, miR-187-5p, miR-188-3p, miR-190a-5p, miR-191-3p, miR-194-3p, miR-195, miR-196a-5p, miR-196b-5p, miR-199a-5p, miR-200a-5p, miR-200b-5p, miR-200c-5p, miR-203, miR-205, miR-204-5p, miR-206, miR-210, miR-211-5p, miR-216a-5p, miR-216b-5p, miR-218-1-3p, miR-218-2-3p, miR-219a-5p, miR-221-3p, miR-223-5p, miR-224-3p, miR-296-3p, miR-297, miR-298, miR-300, miR-302c-3p, miR302a-3p, miR-302d-3p, miR-302d-5p, miR-320a, miR-320d, miR-323b-5p, miR-324-3p, miR-326, miR-330-3p, miR-331-5p, miR-338-3p, miR-338-5p, miR-339-5p, miR-346, miR-363-3p, miR-370-3p, miR-373-3p, miR-373-5p, miR-374b-3p, miR-374a-3p, miR-378a-3p, miR-378a-5p, miR-383-5p, miR-410-3p miR-412-3p, miR-424, miR-425-5p, miR-431-3p, miR-432-5p, miR-449b-3p, miR-449b-5p, miR-454-3p, miR-466, miR-483-5p, miR486-3p, miR-488-5p, miR-493-3P, miR-504-5p, miR-506-3p, miR-509-3p, miR-509-5p, miR-514a-3p, miR-515-3p, miR-516a-5p, miR-517b-3p, miR-518a-3p, miR-518f-5p, miR-519d-3p, miR-520d-3p, miR-520g-3p, miR-524-3p, miR-524-5p, miR-544a, miR-545-5p, miR-548v, miR-550b-3p, miR-556-3p, miR-564, miR-566, miR-567, miR-575, miR-590-3p, miR-590-3p, miR-591, miR-593-3p, miR-593-5p, miR-595, miR-596, miR-597-5p, miR-598-3p, miR-600, miR-601, miR-608, miR-609, miR-612, miR-613, miR-615-3p, miR-616-3p, miR-618, miR-619-3p, miR-622 miR-623, miR-631, miR-632, miR-634, miR-639, miR-640, miR-641, miR-646, miR-648, miR-649, miR-655-3p, miR-662, miR-675-3p, miR-675-5p, miR-708-5p, miR-765, miR-766-3p, miR-770-5p, miR-885-3p, miR-887-3p, miR-888-5p, miR-937-3p, miR-1178-3p, miR-1182, miR-1184, miR-1205, miR-1207-3p, miR-1207-5p, miR-1208, miR-1231, miR-1237-3p, miR-1246, miR-1251-5p, miR-1256, miR-1257, miR-1258, miR-1260a, miR-1264, miR-1266-5p, miR-1267, miR-1269a, miR-1276, miR-1280, miR-1281, miR-1282, miR-1288-3p, miR-1289, miR-1291, miR-1296a-5p, miR-1303, miR-1306-3p, miR-1537-3p, miR-1912, miR-1976, miR-1909-5p, miR-2113, miR-2115-5p, miR-2116-3p, miR-3116, miR-3121-3p miR-3125, miR-3126-5p, miR-3126-3p, miR-3128, miR-3139, miR-3141, miR-3149, miR-3151-5p, miR-3153, miR-3155b, miR-3156-5p, miR-3161, miR-3162-5p, miR-3177-3p, miR-3180-3p, miR-3184-5p, miR-3186-3p, miR-3190-3p, miR-3191-3p, miR-3201, miR-3605-5p, miR-3607-5p, miR-3611, miR-3618, miR-3652, miR-3653, miR-3654, miR-3661, miR-3674, miR-3675-5p, miR-3679-5-p, miR-3682-3p, miR-3685, miR-3688-3p, miR-3689e, miR-3692-3p, miR-3714, miR-3907, miR-3917, miR-3918, miR-3923, miR-3934-5p, miR-4251, miR-4252, miR-4253, miR-4260, miR-4263, miR-4267, miR-4274, miR-4276, miR-4279, miR-4294, miR-4297, miR-4302, miR-4303, miR-4304, miR-4306, miR-4316, miR-4317, miR-4319, miR-4328, miR-4487, miR-4706, miR-4731-5p, let-7a-3p, let-7b-3p, let-7e-3p, let-7f-5p, let-7i-3p.

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