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miRNAs in Pancreatic Cancer

Radu Albulescu, Adrian Claudiu Popa, Elena Codrici,
Daniela Ionela Popescu, Simona Mihai and
Cristiana Tanase

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1. Introduction

Pancreatic cancer and especially PDAC (Pancreatic Ductal AdenoCarcinoma) is among the most difficult to treat cancer, characterized by invasiveness, metastatic potential and bad outcomes.

miRNAs emerged in recent years as potent regulators of cellular activities, playing a central role in controlling the protein expression at the post-transcriptional level. They have significant implication in pathology in general and most relevantly in cancers. Their main role is the control of the process of proteosynthesis at the translational level, by leading their target mRNAs as a miRNA-mRNA dimer to a degradative complex.

Deregulation in expression levels of miRNAs and some genetic alterations were demonstrated in various cancers, including PDAC. Investigations on tissue samples provided a considerable amount of knowledge, leading to the identification of miRNAs with altered expression associated with tumorigenesis and tumor progression. Tumor-inducing and tumor-promoting miRNAs were significantly up-regulated, while sets of tumor-suppressor miRNAs are down-regulated or suppressed. By targeting major protein players in cell regulatory networks, some miRNAs appear to have the ability to shift the balance towards tumorigenesis, while other miRNAs are seen inhibiting or even reversing the process.

Tissular and soluble miRNAs were demonstrated as potential biomarkers, serving as diagnostic, stratification or prognostic tools, while other representatives were identified as "candidate" therapeutic targets or "candidate" therapeutic tools.

MicroRNAs (miRs, miRNAs) form a class of small-sized but powerful cell regulators. Presently, the family of human miRNAs comprises 1872 precursors and 2578 mature forms, but

the discovery process is adding further members at a rapid rate [1, 2]. The conventional nomenclature of miRNAs establishes some rules: a mature miRNA is designated in the form hsa-miR-121, where the first three characters encode the species. The letters that may occur at the end of the name refer to the different locations where the coding gene is located (the same miRNA can be encoded on multiple chromosomes and on either + or – strands). At the same time, the precursors are designated in the form hsa-mir-121. The rate of discovery is quite fast, so usually the numbers are assigned in the sequential order of discovery. However, if a new miRNA has a similar sequence to an existing one, it will acquire identical names, the differentiation being made by the letter. For historical reasons, the first miRNAs discovered, let-7 and lin-4, are exempt from the rule.

miRNAs act in the post-transcriptional regulation of protein expression and their involvement was demonstrated in normal processes as well as in pathology. Most of them are "multivalent", so that one single miRNA is able to "target" multiple genes, thus regulating the expression of several proteins. miRNAs are non-coding RNA molecules, 18-28 nucleotides lengths in the mature form, that regulate a variety of cellular processes including cell differentiation, cell cycle progression and apoptosis. miRNAs can function either as oncogenes or tumor suppressors [3]; oncogenic miRNAs (oncomiRs) are up-regulated in cancer cells [1].

In cancer, several miRNAs are situated "upstream" of the carcinogenesis process – acting as triggers for carcinogenesis or for progression; other miRNAs are situated "downstream" of the carcinogenic process, their modified expression appearing as the outcome of carcinogenetic transformation or progression. miRNAs play major roles in the multistep processes of carcinogenesis, either by oncogenic or tumor-suppressor functions. The study of miRNAs has been extended into many kinds of tumors, including those of the pancreas [4, 5]. Those studies have revealed that miRNAs may be potential diagnostic or prognostic tools for cancer [6, 7]. miRNAs are important tools due to their suitability for detection in both tissues [either fresh or Formalin-Fixed-Paraffin-Embedded (FFPE)] and in other biological samples (blood, serum, plasma, saliva, feces).

The discovery of miRNAs opened new opportunities for non-invasive tests for the early diagnosis of cancer [8, 9]. It has been recently revealed that, once detected, the miRNAs (being differentially expressed in blood) can be used as diagnostic and prognostic circulating biomarkers [10].

In the present chapter we summarize some of the existing knowledge regarding miRNAs involved in tumorigenesis and progression of pancreatic cancer. We have focused on the possible diagnostic role of miRNAs and their tissue-related expression in correlation with their soluble forms. We have summarized recent evidence regarding the assessment of their diagnostic value in pancreatic cancer patients.

2. miRNAs: Biogenesis and mechanisms of action

miRNAs act as post-transcriptional regulators of gene expression in eukaryotic cells. Their biological roles in development, normal cell function and in pathology, including cancer,

have been described and several reviews thoroughly describe the processes involving miRNAs [11, 12].

A brief description of the basic mechanisms of biogenesis may be given as follows:

• miRNAs are encoded in various locations (both in protein-coding and non-coding gene sequences); often, the location of these coding sequences is in fragile chromosomal regions; therefore, they are highly susceptible to molecular modification.

• miRNA sequences are transcribed by RNA polymerase II as larger primary-microRNA molecules, which are further processed by Rnase III endonucleases Drosha and DGCR8 to form precursor miRNAs (pre-microRNAs, stem-loop structures containing about 70 nucleotides).

• Exportin 5 transfers pre-microRNAs to the cytoplasm.

• Processing in the cytoplasm is performed by Dicer (an RNAse III endonuclease), which removes the loop of the pre-miRNA and generates an imperfect duplex, formed by the mature miRNA sequence and a fragment of similar size derived from the opposite side of the loop, (miRNA*).

• The counter strand is separated and most often degraded; however, in many cases this counter strand can also function as a regulator.

Gene expression is controlled by regulation of mRNA translation and degradation:

• Perfect or near-perfect complementarity targets mRNA for degradation by RISC (RNA-Induced Silencing Complex).

• Imperfect complementarity blocks translation by the ribosome.

The majority (if not all) of miRNAs are multivalent. That is, almost every miRNA has the ability to interfere with multiple genes. Often a "cross talk" between miRNAs and other cell-regulatory or effector proteins is encountered, generating a mutual modification of expression, resulting in negative regulatory loops.

A novel pathway, translation activation, was demonstrated by Vasudevan et al. (2007) for miR-369-3. Cell cycle arrest by serum starvation transforms the TNFα AU-rich element (ARE) into a translation activator signal. AGO2 (Argonaute2) and FXR1 (fragile X mental retardation–related protein 1 (FXR1) are associated with ARE on translation activation; both proteins are required to increase translation efficiency. The seed sequence (the nucleotides 2-8 at the 5' end of the miRNA [13] of miR-369-3 was demonstrated to be able to form base-pairs with two target sites on the minimal TNFα ARE required for translation activation. The formation of base-pairs between mir-369-3 and the target sites was demonstrated to be required for translation activation by knock-down experiments and by experiments using mutant ARE, as well as modified sequences of miR-369-3 (in order to restore complementarity to modified targets on mutant ARE) [14].

3. miRNAs in tumor progression

3.1. Cell growth and proliferation

Low levels of expression of miR-34a, 34b and 34c were found in cultivated pancreatic cancer cells (MiaPaCa2 and BxpC3), while the levels of the target genes Bcl2 (Apoptosis regulator Bcl-2) and Notch1 (Neurogenic locus notch homolog protein 1) were elevated. Restoration of miR-34 levels by transfection with miR-34 mimics down-regulation of the target genes, inhibits clonogenic growth and activates apoptosis via the caspase-3 pathway [15].

miR-21 over-expression is demonstrated in PDAC. Its presence and over-expression is associated with poor survival, invasiveness and resistance to gemcitabine. The findings relating to miR-21's role and mechanism in tumor tissue were confirmed *in vitro*, on primary cultures and cancer cell lines, fibroblasts and normal pancreatic ductal cell lines [16]. Enhancement of miR-21 levels (by pre-miR-21 transfection) decreased the anti-proliferative and anti-apoptotic effects of gemcitabine and up-regulated the expression of MMP2 (Matrix-MetalloProteinase 2) and MMP9 (Matrix-MetalloProteinase 9) [16].

3.2. Tumorigenesis

In the case of pancreatic cancer, as is the case in other cancers, distinct patterns of expression of miRNAs occur, depending on disease stage. The expression changes during progression. From these miRNAs, some are common to many cancers, while a few are tissue-specific and can help to track more precisely the tissue in which a carcinogenic process takes place [17]. There is a clear distinction between pre-malignant lesions, primary tumors and metastasis in the pattern of expression of miRNAs. Moreover, some of these distinctions can also be made in exosomal miRNAs.

Deregulated expression of miRNAs may represent an early modification in pancreatic tumorigenesis, generating progression of PanIN (Pancreatic Intraepithelial Neoplasia) lesions to more invasive forms. Ryu et al. investigated three candidates (selected on the basis of previous reports as over-expressed in pancreatic cancer). mir-155 was significantly over-expressed in PanIN-2 and 3 (2.6-fold, p=0.02 and 7.4-fold p=0.049, respectively); miR-21 was over-expressed only in PanIN-3 (2.5-fold, p=0.02), while no modification was found for miR-221 in PanIN lesions compared to normal duct epithelium [18]. Another set of miRNAs were investigated by du Rieu et al. in laser-dissected tissue samples from PanIN lesions (from a mouse model and from human patients). miR-21, 205 and 200 paralleled PanIN progression in mouse models. mir-21 and miR-205 preceded phenotypic changes of the duct. In precursor lesions, miR-21 achieved the highest relative concentrations. In human samples, miR-21,-221, 222 and let-7a increased with lesion grade, with maximal expression in two thirds of lesions. Up-regulation of miR-21 was controlled by KRAS and EGFR in PDAC-(Pancreatic Ductal AdenoCarcinoma) derived cell lines [19].

Another complex investigation of miRNA signatures during tumorigenesis and progression in pancreatic cancer was reported by Olson et al. The study stressed the down-regulation of the miR-200 family in metastases and metastasis-like primary tumors. Also, multiple changes

in microRNA expression in tumor stages were investigated [20]. A synthesis of these data is presented in table 1.

miRNA	Modification	Significance	Ref.
let-7	Down-regulated		[21, 22]
let-7d	Up-regulated		[23, 24]
let-7f-1	Up-regulated		[21]
miR-10a	Up-regulated	Metastasis	[22] [20, 25]
miR-10b	Up-regulated		[25]
miR-15b	Up-regulated	Metastasis, Hyperplasia	[20, 25]
miR-16-1	Up-regulated		[24]
miR-17-5p	Up-regulated	Angiogenesis, Hyperplasia	[20]
miR-18a	Up-regulated		[26]
miR-19b	Up-regulated	Angiogenesis	[20]
miR-20a	Up-regulated	Hyperplasia, angiogenesis	[20]
miR-21	Up-regulated	Angiogenesis	[24, 26]
miR-23a	Up-regulated		[25]
miR-23b	Up-regulated	Metastasis	[20, 25]
miR-24	Up-regulated	Metastasis	[20]
miR-24-1,2	Up-regulated		[24]
miR-25	Up-regulated	Hyperplasia, dysplasia	[20]
miR-27b	Up-regulated	Metastasis	[20]
miR-29c	Down-regulated		[26]
miR-31	Up-regulated		[26]
Mir-92	Up-regulated	Hyperplasia, dysplasia, metastasis	[20]
miR-92-1	Up-regulated		[24]
miR-93	Up-regulated		[26]
miR-95	Up-regulated		[26]
miR-96	Down-regulated		[24]
miR-99	Up-regulated		[25]
miR-100	Up-regulated		[24, 25]
miR-100-1/2	Up-regulated		[25]
miR-103-2	Up-regulated		[25]
miR-106a	Up-regulated	Hyperplasia, dysplasia	[20]
miR-107	Up-regulated		[24, 25]
miR-124a	Up-regulated	Tumor signature, metastasis	[20]
miR-125a	Up-regulated		[25]
miR-125b-1	Up-regulated		[24, 25]
miR-126	Up-regulated	Metastasis	[20]

miRNA	Modification	Significance	Ref.
miR-126*	Up-regulated	Angiogenesis	[20]
miR-129	Up-regulated	Metastasis	[20]
miR-129-3p	Up-regulated	Angiogenesis	[20]
miR-130b	Down-regulated		[24]
miR-132	Up-regulated	Metastasis, tumor signature	[20]
miR-137	Up-regulated	Metastasis	[20]
miR-139	Down-regulated		[24]
miR-141	Down-regulated	Metastasis	[20]
miR-142-p	Down-regulated		[24]
miR-142-3p	Down-regulated	Hyperplasia, angiogenesis, tumor signature	[20]
miR-142-5p	Up-regulated	Angiogenesis	[20]
	Down-regulated	Tumor signature	
miR-143	Down-regulated	Metastasis	[25],18,[26]
miR-145	Down-regulated	Metastasis	[20, 24, 26]
miR-146	Up-regulated		[25]
miR-146a	Down-regulated	Angiogenesis	[20, 26]
		Metastasis	
miR-148a	Down-regulated	Metastasis	[25, 26]
miR-148b	Down-regulated	Metastasis	[25, 26]
miR-150	Down-regulated	Tumor signature	[20, 26]
miR-152	Down-regulated	Metastasis	[20]
miR-155	Up-regulated	Hyperplasia/dysplasia	[20, 25, 26]
miR-181a	Up-regulated	Metastasis	[20, 24, 25]
miR-181b	Up-regulated	Metastasis	[20, 25]
miR-181b-1	Up-regulated		[25]
miR-181b-2	Up-regulated		[25]
miR-181c	Up-regulated		[25]
miR-181d	Up-regulated		[25]
MiR-182	Down-regulated	Metastasis	[20]
miR-184	Down-regulated	Tumor signature	[20]
miR-186	Up-regulated		[27]
miR-189	Up-regulated	Metastasis	[20]
miR-190	Up-regulated		[27]
miR-196a	Up-regulated		[26]
miR-196b	Up-regulated		[26]
miR-199a-1	Up-regulated		[25]
miR-199a-2	Up-regulated		[25]
miR-200a	Down-regulated	Metastasis	[20]

miRNA	Modification	Significance	Ref.
miR-200b	Down-regulated	Metastasis	[20, 27]
miR-200c	Up-regulated	Metastasis	[20]
miR-203	Up-regulated		[26]
miR-205	Up-regulated		[25, 26]
miR-210	Up-regulated		[25]
miR-212	Up-regulated		[24]
miR-213	Up-regulated		[25]
miR-216	Down-regulated		[26]
miR-217	Down-regulated		[26]
miR-220	Up-regulated		[25]
miR-221	Up-regulated		[24-27]
miR-222	Up-regulated		[25-27]
miR-223	Up-regulated		[25]
miR-224	Up-regulated		[26]
miR-301	Up-regulated		[24]
miR-329	Up-regulated	Metastasis	[20]
miR-335	Down-regulated	Tumor signature	[20]
miR-344	Up-regulated	Metastasis	[20]
miR-345	Down-regulated		[24]
miR-365	Down-regulated	Metastasis	[24]
miR-375	Down-regulated		[25, 26]
miR-376a	Up-regulated		[24]
miR-376	Up-regulated	Tumor signature	[24]
409-3p	Up-regulated	Tumor signature	[20]
miR-410	Down-regulated	Hyperplasia	[20]
	Up-regulated	Metastasis	
miR-424	Up-regulated	Angiogenesis	[20, 24]
miR-429	Down-regulated	Metastasis	[20]
miR-431	Up-regulated	Metastasis	[20]
miR-434-3p	Up-regulated	Tumor signature	[20]
miR-449	Up-regulated	Metastasis	[20]

Table 1. miRNAs involved in tumorigenesis of the pancreas

3.3. Apoptosis, cell viability

miR-21, miR-155 and miR-221 over-expression was reported by Lee et al. (2007) for pancreatic tumors compared to paired normal samples. Since the same miRNAs were also over-expressed in other cancers, the authors hypothesized that deregulation of these miRNAs represents a common feature in cancer. For other miRNAs, the pattern of differential expression appeared

different in pancreatic cancer compared to other cancers; modification of miR-376a and miR-301 expression was reported as a distinctive feature of pancreatic cancer [24].

Inhibiting miR-21 and miR-221 with antisense nucleotides resulted in reduced proliferation and increased apoptosis [28].

Hanoun et al. reported the identification of 29 miRNA encoding genes that are susceptible to inactivation by hypermethylation. "In-depth" investigations on miR-148a showed that its production was repressed due to hypermethylation. Hypermethylation analysis was demonstrated as a potential tool for differential diagnostics for PDAC and pancreatitis [29].

Down-regulation of miR-146 was demonstrated by Li et al. (2010) in pancreatic cancer cells compared with normal duct epithelium. Re-expression of miR-146 inhibits the invasive capacity of cancer cells, concomitant with down-regulation of EGFR (Epidermal Growth Factor Receptor) and IRAK-1 (Interleukin 1 Receptor-Associated Kinase). Treatment with two natural compounds, diindolylmethane and isoflavone, were demonstrated to activate miR-146a and inhibit invasion [30].

3.4. Tumor suppressors

Mees et al. (2009) applied microarray, TLDA (Taq-Man Low Density Array) and RT-PCR (Real Time Polymerase Chain Reaction) methods to investigate microRNA profiles in pancreatic cancer cell lines. Fifty-six miRNAs with modified expression were identified: 27 (by microarray) and 19 (by TLDA) miRNAs were over-expressed in highly metastatic cell lines compared to less metastatic ones. Down-regulation (investigated by TLDA) revealed 35 down-regulated microRNAs. Eight of these were tumor-suppressor gene-related miRNAs: miR-21 (PTEN-Phosphatase and TENsin Homolog), miR-26b (EP300-E1A binding protein p300, PTEN), miR-194 (EP300), miR-200b (EP300), miR-200c (EP300), miR-320 (PTEN), miR-374 (EP300) and miR-429 (EP300) [31].

The influence of miR-10a on the behavior of pancreatic tumors was investigated by Weiss et al. in a zebrafish animal model (zebrafish with transplanted tumors) [22]. miR-10a promotes metastatic potential and miR-10 repression is sufficient to inhibit invasions and metastasis. mir-10a is a retinoic acid (RA) target and RA receptor antagonists are effective repressors of miR-10a expression. The anti-metastatic effect is blocked by the knockdown of HOXB1 (Homeodomain containing DNA-binding Box protein 1) and HOXB3 (HOXB3=Homeodomain containing DNA-binding Box protein 3) genes. The epithelial to mesenchymal cell transition (EMT) program triggers cellular mobility and promotes invasion and metastasis. ZEB1 (zinc finger E box binding homeobox1), an EMT activator, promotes cell mobility by disrupting stemness maintenance and promoting mobile, migrating stem cells. ZEB1 was demonstrated to inhibit miR-200 family members and miR-203 [32].

A specific miRNA signature differentiates between pancreatic adenocarcinoma, normal pancreas and chronic pancreatitis [25]. In total, 21 over-expressed and four down-regulated miRNAs allow a differential diagnosis among these three pathologic conditions. In addition, Szafranska et al. reported that miR-196a and-196b levels are high in pancreatic ductal adenocarcinoma but not in normal or inflamed pancreatic tissues [26].

4. miRNAs in tumor stem cells

In gemcitabine-resistant cells with fibroblast morphology, high levels of vimentin and ZEB1 and low levels of E-cadherin, miR-200b, miR-200c, let-7b, 7c, 7d and 7e were found to be down-regulated, according to Li et al. (2009). As in the case of miR-146, DIM (3, 3' DiIndolylMethane) and isoflavone were demonstrated to restore a less invasive phenotype [33]. ZEB1 was demonstrated to repress expression of stemness-inhibiting miR-203; candidate targets of miR-200 family members are also stem cell factors, such as Sox2 (Transcription factor SOX-2) and Klf4 (Krueppel-like factor 4). miR-200c, miR-203 and miR-183 cooperate to suppress expression of stem cell factors in cancer cells and mouse embryonic stem (ES) cells, as demonstrated for the polycomb repressor Bmi1 (Polycomb complex protein BMI-1) [32].

Key cell differentiation programs during development are controlled by the members of let-7 and miR-200 families. In cancer, loss of let-7 leads to disease progression and de-differentiation [33]. The same let-7 family appears as a regulator of EMT and of stem cell maintenance. The EMT process is regulated by miRNA-dependent mechanisms. In human pancreatic cancer, DCLK1 (Serine/threonine-protein kinase DCLK1) regulates EMT by a mechanism dependent on miR-200a [33-35]. According to Hasselman et al. [36], inhibition of maturation of let-7 by nuclear TRAIL-R2 (TNF-Related Apoptosis-Inducing Ligand Receptor 2) in pancreatic cancer cell lines increases their proliferation. This is consistent with high levels of nuclear TRAIL-R2 in tissue samples from poor outcome patients [36].

The population of BxPC-3-LN cells (lymph node metastatic pancreatic cells) contains a five-fold increased population of CD133+/CXCR4+cells (stem cell-like cells) compared with the parental (non-metastatic) BxPC-3 cells. Remarkably, a different miRNA pattern is displayed in CSC-like cells compared with the regular cells: up-regulated miR-572, miR-206, miR-449a, miR-489 and miR184 were found, as well as down-regulated let-7g-3p, let-7i-3p, let-7a-3p, miR-107, miR-128 and miR-141-5p [37].

The miR-200 family members are identified as key regulators of cell maintenance and EMT. It is considered possible that tumor progression is a process resulting in progressive de-differentiation towards a cell type which has a stem cell-like phenotype. This process appears to be regulated by miRNA-dependent mechanisms. DCLK1 (a putative marker for pancreatic and intestinal cancer stem cells) regulates EMT in human pancreatic cancer cells via a miR-200a-dependent mechanism [38]; it also acts as a regulator of let-7a in pancreatic and colorectal cancer cells, supporting the idea that these miRNAs may be novel and relevant targets in solid tumor cancers [33-35]. Sureban et al. [39] demonstrated that DCLK1 inhibition results in up-regulation of miRNAs that negatively regulate some key angiogenic and pluripotency factors. In AsPC1 (metastatic adenocarcinoma cell line) tumor xenografts, the down-regulation of c-MYC (Myc Proto-oncogene Protein) and KRAS (GTP-ase Kras) via let-7a was observed, by a similar mechanism demonstrated in pancreatic cancer cells.

5. miRNA Polymorphisms

Single nucleotide polymorphisms (SNPs) were demonstrated to affect the functional capacity of miRNAs, influencing MIR processing and miR-mRNA interactions. SNPs in miR-196a2 and miR-146a were differentially expressed between patients with T1/T2 stage pancreatic tumors compared with T3/T4 stages [40].

6. Circulating miRNAs

Some serum tumor markers, such as carcinoembryonic antigen and carbohydrate antigen 19–9, are used as convenient diagnostic markers. Other factors involved in cancer progression, among which are angiogenic factors such as VEGF (Vascular Endothelial Growths Factor) and bFGF (Basic Fibroblast Growth Factor), have drawn attention for the detection of pancreatic cancers [41, 42]. However, these conventional serum markers lack the sensitivity and specificity to facilitate the early detection of cancer. Several studies have identified tumor-specific alterations in plasma/serum nucleic acids in cancer patients and have shown the potential of plasma-circulating nucleic acids to act as new non-invasive biomarkers in patients with various cancers [43]. Recently, several studies have demonstrated that miRNAs are stably detectable in plasma/serum and have discussed key aspects regarding experimental design, such as extraction from biological material, different techniques for miRNA evaluation (TLDA, arrays, etc.) [44-46]. Mitchell et al. clearly showed that circulating miRNAs originate from cancer tissues and are protected from endogenous RNase activity. They also demonstrated that the circulating plasma miRNAs are not associated with circulating tumor cells [45].

Li et al. investigated a set of 735 miRNAs by RT-qPCR (Reverse-Transcriptase quantitative Polymersase Chain Reaction) using microarrays. Eighteen candidates were further validated. The best classifier was miR-1290, with ROC-AUC (Receiver Operator Characteristics Area Under the Curve) of 0.96, while other miRNAs (miR-24, miR-134, miR-146a, miR-378, miR-484, miR-628-3p and miR-1825) also displayed considerable accuracy (ROC-AUC > 0.7). miR-1290 could differentiate between normal pancreas, chronic pancreatitis, pancreatic adenocarcinoma and pancreatic neuroendocrine tumors. Remarkably, miR-1290 is a better classifier than the classical biomarker CA 19-9, distinguishing with greater accuracy low-grade pancreatic cancer from normal subjects [47].

Morimura et al. demonstrated the value of miR-18a as a biomarker for pancreatic cancer; they demonstrated higher levels of expression of this miRNA in cancer tissue and cancer cell lines (compared to normal tissue) and also reported higher plasma levels in patients with PC, with ROC-AUC of 0.9369 [46].

A signature of seven miRNAs was established as a good biomarker for early detection by Liu R et al. [48]. The panel comprised miR-20a, miR-21, miR-24, miR-25, miR-99a, miR-185 and miR-191; the levels of overexpression in plasma ranged between 2.1 and 5.08.

Exosomal miRNAs represents a more recent field of investigation. Exosomes are 40-100 nm vesicles derived from the fusion of multivesicular bodies with the plasma membrane. They

appear in all body fluids and interest in studying them has increased since they contain functional proteins, mRNA and miRNAs. Thus, exosomal populations of different origins may be identified by their protein and miRNA signatures. Moreover, they appear to be actively involved in cell communication. In the case of cancer, they will be, for instance, involved in tumorigenesis, differentiation of stem cells, metastasis and angiogenesis [49]. Most of the reports on exosomes so far concern other cancers, like ovarian [50], prostate [51] and glioblastoma [52]; however, there is also a study concerning exosomes in pancreatic cancer [53].

7. miRNAs: Therapeutic targets and drugs

miRNAs, already described as potent regulators of genes, can be viewed as both therapeutic targets and therapy agents [54]. Recently, the potential to target miRNAs was demonstrated by a series of *in vitro* studies.

In pancreatic cancer (and other epithelial tumors as well), a loss of epithelial differentiation and acquisition of the mesenchymal phenotype occurs, leading to enhanced invasion and migration [55]. Another feature of pancreatic cancer is its drug resistance characteristics, often associated with epithelial-to-mesenchymal transition (EMT) [56].

miR-21 overexpression is associated with resistance to gemcitabine and is generally associated with poor survival [57]. Inhibition of miR-21 decreases cell proliferation and promotes apoptosis [58] but also correlates with 5-FU (5-Fluoro-Uracyl) sensitivity [59]. Another study points out that miR-200 down-regulation and over-expression of miR-21 associates with gemcitabine resistance and their restoration renders the pancreatic cell lines responsive to gemcitabine [60].

miR-34 is a tumor-suppressor miRNA, which can restore chemo-and radio-sensitivity in tumor cell lines; overexpression of miR-34 reduces the tumor-initiating cells and tumor-sphere formation significantly [61].

Another candidate is miR-155, which also appears down-regulated in pancreatic cancers; its overexpression appears to suppress tumor growth [18, 62]. Similar findings are also published for miR-20 and miR-146, with regard to their impact on invasiveness and chemo-sensitivity [63, 64].

The published literature dealing with miRNAs as therapeutic targets in digestive tract cancers does not abound and relies mostly on results obtained on cell lines. miRNAs as therapeutic targets are foreseen in chemotherapy resistance [65-67], silencing oncogenic miRNAs and intervention on tumor-suppressive miRNAs.

Another important set of studies focuses on miRNAs' oncogenic function and on the modalities of intervening using miRNA silencing, antisense blocking and miRNA modifications [54].

The miRNAs with tumor-suppression functions can represent new strategies for inhibiting tumor growth in pancreatic cancer, liver cancer and colorectal cancer [68], while miRNAs as oncogenes can be targeted leading to controlling multiple genes [69].

Recently, the inhibition of miR-21 and miR-17-92 activity was reported as being associated with reduced tumor growth, invasion, angiogenesis and metastasis in PDAC [70, 71].

As therapeutic targets, miRNAs can be manipulated with silencing methodology or recovery of altered microRNAs. Using miRNAs as therapeutic targets may result in several clinical goals: prevent recurrence of the disease, control the growth of advanced metastatic tumors and sensitize tumor cells to chemo-and radiotherapy. There are few studies on *in vivo* experimental models and no clinical trial have commenced using these small molecules as targets [54]. Thus, miRNAs may be possible drugs and/or drug targets and they could potentially be used as molecular therapeutic agents that could inhibit oncogenes or restore the expression of silenced tumor-suppressor genes [1, 72].

Taking into account the updated findings, miRNA-based cancer gene therapy is to be used as follows: RNA or DNA drugs against messenger RNA-encoding genes involved in the pathogenesis of cancers or by directly targeting ncRNAs (non-coding RNAs) that participate in cancer pathogenesis, as reported in colorectal cancers [73].

The reported stages of miRNAs as drugs are generally at the preclinical phase. Groups are using cell lines or even primary cells in a workflow comprising *in vitro* treatment and afterwards detecting the alteration of proliferation, increase in apoptosis and/or abolishment of cancer stem cell characteristics. In animal models treating tumor-bearing mice with specific siRNA, the overall effect on tumor development and survival was tested along with the excretion route of the drug. Very few clinical studies are reported and mostly only in phase I [74, 75]

Up to now, the reported inhibitory RNAs drugs have been: antisense oligonucleotides (ASOs), ribozymes or DNAzymes, siRNAs, microRNAs mimetics, LNAs (Locked Nucleic Acids), anti-miRNAs or antagomiRs (small synthetic oligonucleotides blocking the binding of miRNAs to their targets [28, 76].

siRNAs represent a double strand RNA homologous to the mRNA of a target gene. These siRNAs are incorporated into a multiprotein RNA-induced silencing complex (RISC). The antisense strand guides this complex to its homologous mRNA target for endo-nucleasic cleavage of messenger RNA. ERBB2 (ErbB2 protein encoding gene) amplification was demonstrated in gastrointestinal adenocarcinomas, while in cellular and animal models, siRNA was used to knock down ERBB2 in cell lines, demonstrating that this treatment decreased ERBB2 protein levels and apoptotic pathways were triggered [77].

siRNA specific for bcl-2 (Apoptosis regulator Bcl-2) was also used as a possible therapeutic tool in pancreatic cancer in cells lines and xenografts and the bcl-2 gene was inhibited [78].

MicroRNAs mimics represent small single-strand 19–24 nucleotide RNA produced from the cleavage of a hairpin structure by RNAse III enzymes. These possible therapeutic agents act by the inhibition of protein production by either mRNA degradation or translational block after the formation of miRNA<mRNA duplexes.

miR-34 can target p53, Notch, HMGA2 (High Mobility Group Protein HMGI-C) and Bcl-2, genes mainly involved in cancer stem cells characteristics. There are no clinical trials published

so far of these possible drugs, therefore only preclinical studies were reported, showing that upon the insertion of a functional miR-34, inhibition of cell growth, chemo-sensitization and apoptosis are triggered along with the abolishment of cancer stem cell characteristics [79].

8. Conclusions

Modification in the expression of miRNAs is consistently associated with the process of tumorigenesis. Such deregulation of miRNAs encompasses early-stage or tumor-initiating events, triggers invasion and metastasis, or alternatively it may also represent the outcome of complex alterations specific to tumor cells. Deregulated miRNAs were demonstrated to have potential and several have been already validated as biomarkers for cancer diagnostics or prognosis, including several for pancreatic cancer, especially in tissue-based investigations.

A great number of miRNAs have similar expression patterns in cancers, but several have been demonstrated to be tumor-specific.

A considerable effort is directed towards the development of miRNA-based instruments for diagnostics, prognostics or monitoring the disease and great hope is placed in the exosomal miRNAs. Assays based on exosomal or plasma miRNAs have potential clinical uses in screening patients at risk of cancer or monitoring recurrence post-resection.

They also prove useful in evaluating the completeness of tumor resection and the evaluation of adjuvant therapy. As biomarkers, they show important advantages over other nucleic acids. Compared to the mRNA, in the case of miRNAs a considerably smaller number of molecules can establish an effective screen to differentiate normal from tumoral disease. At the same time, circulating miRNAs are more stable for detection, by comparison with other classes of markers, like mRNAs or proteins.

Meanwhile, the presence of specific miRNAs in pathological tissue opens a new perspective in therapy. As has already been proved, targeting deregulated miRNAs with specific instruments, like miR-mimics, antago-miRs or miRNAs, restores the phenotype from tumoral to normal and the results so far suggest that controlling the expression of miRNA modifies clinical features of tumor cells, such as growth rate, apoptotic susceptibility, drug resistance, mobility and invasiveness of metastatic potential.

9. Abbreviations

Proteins:

AGO2=Protein Argonaute2

ARE=TNFα AU-rich element

Bcl2=Apoptosis regulator Bcl-2

bFGF=Basic Fibroblast Growth Factor

Bmi1=Polycomb complex protein BMI-1

c-MYC=Myc Proto-oncogene Protein

DCLK1=Serine/threonine-protein kinase DCLK1

DGCR8=Integral membrane protein DGCR2/IDD

EGFR=Epidermal Growth Factor Receptor

EP300=E1A binding protein p300

ERBB2=ErbB2 protein encoding gene

EMT=Epithelial-Mesenchymal Transition

FXR1=Fragile X Mental Retardation–related Protein 1

HMGA2=High Mobility Group Protein HMGI-C

HOXB1=Homeodomain containing DNA-binding Box protein 1

HOXB3=Homeodomain containing DNA-binding Box protein 3

IRAK-1=Interleukin 1 Receptor-Associated Kinase

Klf4=Krueppel-like factor 4

KRAS=GTP-ase Kras

MMP2=Matrix-MetalloProteinase 2

MMP9=Matrix-MetalloProteinase 9

Notch1=Neurogenic locus notch homolog protein 1

PTEN=Phosphatase and TENsin homolog

Sox2=Transcription factor SOX-2

TRAIL2=TNF-Related Apoptosis-Inducing Ligand 2

TRAILR2=TNF-Related Apoptosis-Inducing Ligand Receptor 2

VEGF=Vascular Endothelial Growths Factor

ZEB1=Zinc finger E box Binding homeobox1

Other Abbreviations

5-FU=5 Fluoro-Uracil

CSC=Cancer Stem Cells

DIM=3, 3' DiIndolylMethane

FFPE=Formalin-Fixed, Paraffin Embedded

LNA=Locked Nucleic Acids: conformationally-restricted nucleic acid analogue in which the ribose ring is 'locked' with a methylene bridge connecting the 2′-O atom with the 4′-C atom.

PanIN=Pancreatic Intraepithelial Neoplasia

PDAC=Pancreatic Duct AdenoCarcinoma

RA=Retinoic Acid

RISC=RNA-Induced Silencing Complex

ROC-AUC=Receiver Operator Characteristics Area Under the Curve

SNPs=Single Nucleotide Polymorphisms

TLDA=Taq-Man Low Density Array

Author details

Radu Albulescu[1,2], Adrian Claudiu Popa[3], Elena Codrici[1], Daniela Ionela Popescu[1], Simona Mihai[1] and Cristiana Tanase[1]

1 National Institute of Pathology "Victor Babes", Bucharest, Romania

2 National Institute for Chemical Pharmaceutical R&D, Bucharest, Romania

3 Army Center for Medical Research, Bucharest, Romania

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