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Review

MIR376 family and cancer

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Summary. MicroRNAs (miRNAs) are endogenous non-coding small RNAs that negatively regulate gene expression at the post-transcriptional level. They have been implicated in several fundamental biological processes including development, differentiation, apoptosis and stem cell maintenance. There is increasing evidence that microRNAs also play roles in cellular transformation and carcinogenesis by acting either as tumor suppressors or oncogenes. Recent studies introduced *MIR376* as an important microRNA family for cancer formation and progression. The *MIR376* family is located on human chromosome 14 and it has several members containing identical or similar seed sequences. Biological roles of family members were studied in different cancer settings, including gliomas, leukemia, breast and ovarian cancers. Furthermore, two *MIR376* family members, namely *MIR376A* and *MIR376B* were implicated in the regulation of macroautophagy (autophagy herein). Since autophagy dysregulation underlies various diseases including cancer, it is essential to understand the role of the *MIR376* family in this context. In this article, we summarize the miRNA-cancer connection, and review accumulating data about the involvement of the *MIR376* family in cancer biology.

Key words: MicroRNA, *MIR376* family, *MIR376A*, *MIR376B*, Cancer, Autophagy, Cell death

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Introduction

Post-transcriptional modification of gene expression can be achieved through mechanisms involving small RNAs. MicroRNAs (miRNAs) are a subgroup of endogenously expressed non-coding small RNAs. They silence genes through binding protein-coding messenger RNAs (mRNA). MiRNAs affect the half-life of mRNAs, inhibit their translation to proteins, or sequester them and prevent their translation. In this manner, miRNAs downregulate target protein levels in cells. Frequently, miRNAs target dozens of genes, orchestrating several related pathways at once. In addition to their physiological roles, miRNA dysregulations are implicated in diseases such as cancer and neurodegenerative disorders. MiRNAs can either be up- or down-regulated in different cancer types and play tumor suppressor or oncogenic roles according to the cancer setting. Moreover, tumor-related microRNAs may be found in blood circulation and body fluids, allowing them to be used as disease biomarkers.

MiRNA families that have been studied extensively in a cancer setting include the *MIR17-5P* cluster and *LET7* family. The *MIR376* family islocated on the human chromosome 14q32 and at the distal end of mouse chromosome 12, in loci called *Dlk-Dio3* in humans and *Dlk1-Gtl2* in mice, respectively. Alterations in the intracellular levels of *MIR376* family members were found in a spectrum of malignancies, including melanomas, osteosarcomas, ovarian cancers and breast cancers, opening the way for their usage as biomarkers and possibly as gene therapy targets.

In this review, the current knowledge about the *MIR376* family and the role of its members in different

cancer settings will be discussed.

First, we will briefly summarize miRNA pathways and miRNA-cancer relationships, and then we will focus on findings implicating the *MIR376* family in cancer initiation and progression. Additionally, since *MIR376* family members *MIR376A* and *MIR376B* have been implicated in autophagy regulation, following a brief introduction to the relationship between autophagy and cancer, we will explain the role of these miRNAs in autophagy regulation in a cancer setting.

MicroRNAs and their biogenesis

Out of approximately 3 billion nucleotides, only about 2-5% of the human genome codes for proteins. Recent studies showed that up to 90% of DNA that was once considered as "junk" was in fact transcribed and products were not proteins, but RNAs. Among them, miRNAs make up a important class of non-coding RNAs. They function as endogenous regulators of gene expression in various organisms, including plants and animals (Bartel, 2004). It is estimated that miRNAs regulate nearly 30% of human genes, and they are implicated in important biological processes, including differentiation, development, proliferation, cell cycle and cell death. Gene regulation involves targeting of more than one messenger RNA by a single miRNA, allowing coordination of complex genetic networks (Kim, 2005).

Mammalian genomes encode hundreds of miRNA genes that can be located in either intronic or intergenic regions. Intronic miRNAs are transcribed from the intron region of a protein-coding gene (usually a gene belonging to a pathway controlled by the miRNA), whereas intergenic miRNAs are controlled by their own independent promoters. Moreover, the latter might form gene clusters, having more than one separate but homologous miRNAs in the cluster, or they might exist as single genetic units. Sometimes, miRNAs belonging to the same family may be transcribed as long polycistronic transcripts.

In the canonical miRNA biogenesis pathway, miRNAs are first transcribed by RNA polymerase II or III as primary-miRNA transcripts (pri-miRNAs) (Fig. 1). They are then processed in the nucleus by a complex

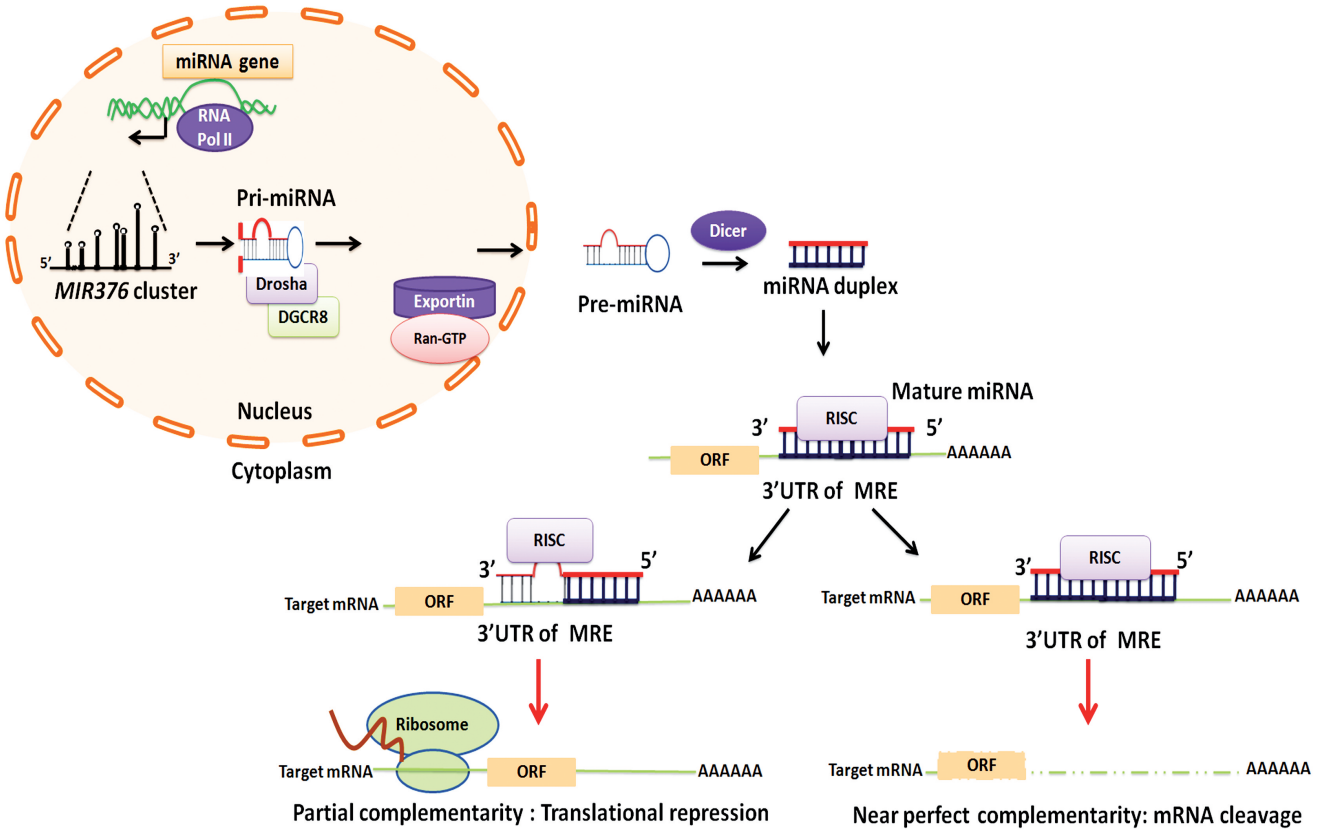


Fig. 1. Canonical microRNA biogenesis pathway in mammals. miRNAs are transcribed by RNA polymerase II and processed by a Drosha-DGCR8 complex in the nucleus. In the cytoplasm, Dicer complex cleaves the hairpin of pre-miRNA to form the miRNA duplex. One of the mature miRNA strands is loaded onto the RNA Induced Silencing Complex (RISC). The fate of the target is determined by the degree of complementarity between the mature miRNA strand and the target mRNA sequences (mainly in the 3'UTR regions). See relevant section in the manuscript for further details.

including Drosha and its regulatory subunit DGCR8, to generate hairpin structured premature-miRNAs (pre-miRNAs) of 60-70 nucleotides. After their transport from nucleus to the cytoplasm by an Exportin-5 complex, DICER protein further cleaves the hairpin structure of pre-miRNAs, leading to the formation of ~21-22 nucleotide long miRNA duplexes. From these duplexes, one of the mature miRNA strands is selected according to its thermostability and loaded onto a complex called the RNA Induced Silencing Complex (RISC). Argonaute (AGO) proteins are components of the RISC and they guide single-stranded mature miRNAs to their target messenger RNAs (mRNAs). The degree of complementarity between mature miRNA seed sequences (~8 nucleotides in the core region of the miRNA) and "miRNA response elements (MRE)" on target mRNA sequences determines target specificity and mRNA fate. MREs are in generally found in the 3'UTR region of the mRNA; however, miRNA binding to the 5'UTR or exonic regions have also been reported. A perfect or near-perfect complementarity between the mature miRNA seed and mRNA target sequences may lead to target mRNA de-capping and/or de-adenylation, followed by its degradation. However, a partial complementarity may block the translation machinery and result in translation inhibition. Another possible regulation mechanism involves sequestration of the miRNA-mRNA complex in P-bodies (Graves and Zeng et al., 2012; Ha and Kim et al., 2014).

Role of microRNAs in cancer

Analysis of miRNAs in different cancer types revealed changes in the level of these small RNAs, and opened the way to their analysis as tumor suppressors and oncogenes (or oncomiRs) as well as cancer biomarkers (Calin and Croce, 2006; Wiemer, 2007).

For example, the *MIR17-92* cluster was over-expressed in several cancer types, including lymphomas, breast, lung, colon and pancreas cancers. Cluster members promoted cancer development through negative regulation of tumor suppressor genes controlling differentiation or apoptosis (Zhang et al., 2007; Ventura et al., 2008). Further evidence suggested that cluster members also played a role in tumor angiogenesis (Wang and Olson, 2009). On the other hand, miRNAs such as *LET7* were down regulated in cancers. *LET7* was shown to target certain oncogenes, and experimental data indicated a role for this miRNA as a tumor suppressor. Similarly, tumor suppressive roles of *MIR181* family members were reported in some cancer types, including gliomas and non-small cell lung carcinomas (Shi et al., 2008; Huang et al., 2015). Another miRNA family that has beem studied thoroughly in a cancer context is the *MIR34* family. Downregulation of *MIR34* family members in breast and colon cancers was observed, and overexpression of miRNAs belonging to *MIR34* family in tumor cells induced cell cycle arrest and apoptosis. Hence, analysis of miRNA expression profiles and functions in cancer can be most rewarding, allowing the discovery of tumor biomarkers for diagnosis and follow-up. Moreover, advances in gene delivery tools might soon allow clinical usage of miRNAs and their inhibitors as novel cancer therapeutics (Jansson and Lund, 2012).

MIR376 family

In 2004, Seitz et al. carried out a computer based approach and identified novel miRNA genes that were located on human chromosome 14q32, at the *Dlk-Dio3* locus. Similar sequences were found on the distal end of the mouse chromosome 12, in the *Dlk1-Gtl2* locus. The genomic loci in question hosted more than 40 potential miRNA genes including the *MIR376* family (Seitz et al., 2004). In addition to miRNA genes, these loci also contained at least three other genes, namely *Dlk1, Rtl1* and *Dio3*. Dlk1 was shown to encode a transmembrane glycoprotein that functionally plays a role in differentiation of several tissues, including adipose tissue (Moon et al., 2002; Lee et al., 2003). The second gene, *Rtl1*, was a retrotransposon-like gene that is expressed during embryonic stages and is necessary for normal placental development (Sekita et al., 2008). On the other hand, the *Dio3* gene encodes for a protein called Type 3 iodothyronine deiodinase, an enzyme that is responsible for the degradation of thyroid hormone and that is also active in placenta (Galton et al., 1999). Moreover, the importance of loci in the 14q32 region for embryonic development was confirmed in mouse studies where severe phenotypes were observed when genetic modifications were made in the gene cluster (da Rocha et al., 2008). The *MIR376* gene family on 14q32 consists of several related miRNAs, namely *MIR376A1-A2, MIR376B, MIR376C* (previously named as *MIR368*), *MIR654* and less characterized putative miRNAs *MIRB1* and *MIRB2* (Kawahara et al., 2007) (Fig. 2). Seed sequences of these miRNAs are either identical (*MIR376A1-A2* and *MIR376B*) or very similar (single base difference among all miRNAs). *MIR654* is the exception, since it harbours a completely different and unrelated seed sequence. MiRNAs with similar seed sequences might share targets and they might be functionally related.

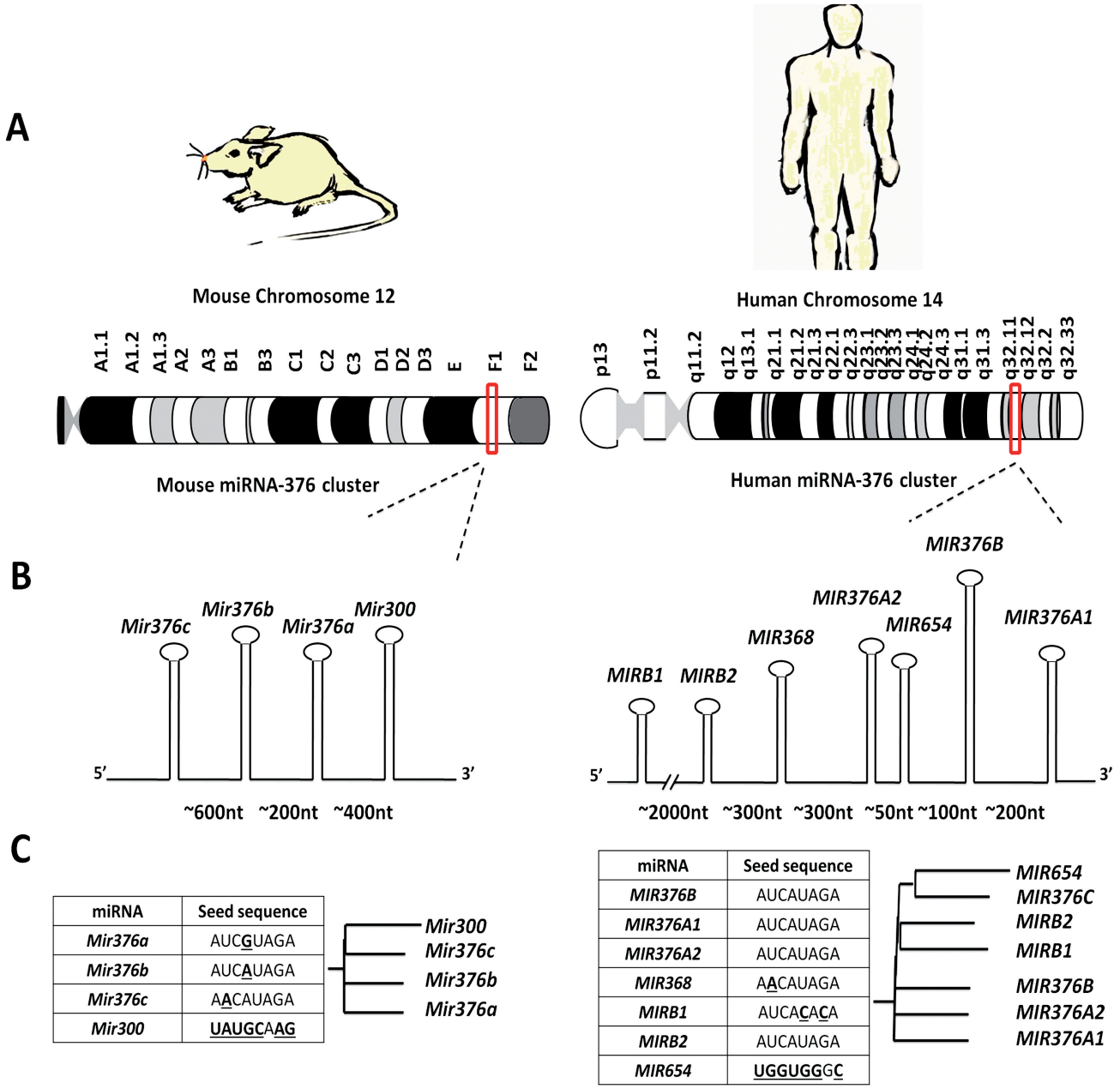
In line with this, accumulating data indicate that some *MIR376* cluster members have overlapping target profiles (see below). Yet, sequences outside the seed sequence were also reported to affect target specificity (Sun et al., 2010).

Members of miRNA gene clusters can be transcribed as long primary transcripts, encoding for several miRNA members. Production of a long transcript from the *MIR376* cluster and presence of individual miRNAs in a common transcript were suggested by a number of studies (Seitz et al., 2004; Teferedegne et al., 2010). Conversely, there is also evidence of independent

regulation of individual miRNAs from this cluster under different stress conditions (Korkmaz et al., 2013). Recently, a transcription factor called AIRE was identified in medullary thymic epithelial cells as one of the transcriptional regulators of *MIR376* family members, including *MIR376A, MIR376B, MIR376C* (Macedo et al., 2013).

RNA editing of *MIR376* family members

RNA editing is a type of post-transcriptional modification, allowing a site-specific and irreversible modification of RNA sequences (Levanon et al., 2004). Modification of adenosine to inosine (A-to-I) is a commonly observed form of RNA editing during which



miRNA	Seed sequence
Mir376a	AUC**G**UAGA
Mir376b	AUC**A**UAGA
Mir376c	A**A**CAUAGA
Mir300	**UAUGC**A**AG**

miRNA	Seed sequence
MIR376B	AUCAUAGA
MIR376A1	AUCAUAGA
MIR376A2	AUCAUAGA
MIR368	A**A**CAUAGA
MIRB1	AUCA**C**A**C**A
MIRB2	AUCAUAGA
MIR654	**UGGUGG**G**C**

Fig. 2. Schematic representation of mouse and human MIR376 family members. **A.** Localization of mouse and human MIR376 family gene cluster on chromosome 12 and 14, respectively. **B.** *MIR376* family members shown as miRNA clusters in mouse (left) or human (right) genomes. **C.** Tables comparing the seed sequences of mouse (left) or human (right) *MIR376* family members. Non-identical residues are marked as bold and underlined. Dendrograms show evolutionary relations between *miRNA* family members. Sequences were analyzed using the ClustalW tool.

deamination of adenosine to inosine occurs in RNA precursors. Proteins from the Adenosine deaminases acting on RNA (ADAR) family regulate the editing process (Bass, 2002). The modification is observed both in vertebrates and invertebrates, and it is important for normal development (Higuchi et al., 2000; Palladino et al., 2000). Changes in the expression patterns of ADARs and a decrease in A-to-I editing were reported in cancers, including gliomas (Paz et al., 2007). In miRNAs, editing mostly occurs in seed sequences; thus it may alter target specificity.

A-to-I editing of *MIR376* family members was reported by Kawahara et al. (2007) and seed sequence changes were reported in transcripts in human cortex and medulla (Kawahara et al., 2007). Another study that was based on massively parallel sequencing and bioinformatics analysis of developing mouse brain transcripts documented that seed editing levels were increased during development for several miRNAs, including *MIR376A1* and *A2* (Ekdahl et al., 2012). As expected, A-to-I editing of the seed region was shown to be critical for target specificity. Kawahara et al. (2007) reported that edited *MIR376A** differentially targeted *PRPS1*, (an enzyme involved in the uric acid synthesis pathway) but the unedited counterpart did not do so (Kawahara et al., 2007). In another study, Choudhury et al. revealed that RNA editing played a role in target preference of *MIR376A** in glioma cells (Choudhury et al., 2012). Microarray profiling of genes that were affected by edited versus unedited *MIR376A** led to the identification of *AMFR* (autocrine motility factor receptor) and a RAS family member, *RAP2A* as differentially expressed transcripts changing the invasive behavior of cancer cells. These data underline the importance of *MIR376* family editing in a tissue-specific manner and indicate that there might be implications for human diseases.

MIR376 family in health and disease

Tissue-specific expression variations are present among *MIR376A, MIR376B* and *MIR376C* members under physiological settings (http://www.mirnabodymap.org/) (Mestdagh et al., 2011). For example, *MIR376A* was found to be highly expressed in brain, retina and uterus, while *MIR376B* was most abundantly expressed in spleen and adrenal glands, and *MIR376C* in the ovaries (Loscher et al., 2007; Choudhury et al., 2012).

In fact, expression levels of most of the *MIR376* family members were high in tissues such as cervix, testicles, placenta, adrenal glands and brain (http://www.mirnabodymap.org/) (Mestdagh et al., 2011). Although *MIR376A* was described as a pancreatic islet-specific microRNA in MIN6 pancreatic beta cell line, further tests showed that it had no effect on basal or glucose stimulated insulin secretion (Poy et al., 2004).

Tissue and cellular levels of *MIR376* family members have been reported to change in response to various physiological signals. Several independent studies provided evidence about the importance of *MIR376* family members for embryonic implantation, development and differentiation. For example, *MIR376A* was reported to be a critical factor for erythrocyte (Wang et al., 2011) and keratinocyte differentiation (Hildebrand et al., 2011) and skeletal muscle development (McDaneld et al., 2009).

Another study showed that *MIR376* cluster members, especially *MIR376A, MIR376B* and *MIR376C*, were present in mouse embryonic inner ears and they were detected in the sensory epithelia and ganglia of both auditory and vestibular portions (Yan et al., 2012). *MIR376C* was found to be downregulated in implantation sites compared to interimplantation sites in early pregnancy, suggesting a regulatory role for the miRNA in the process (Geng et al., 2014). The atypical member of the *MIR376* family, *MIR654*, was found to be expressed in follicular fluid of bovine oocytes (Sohel et al., 2012) and it was upregulated in premature ovarian failure (POF) patients (Yang et al., 2012). Similarly, *MIR376A* was expressed in embryonic and neonatal ovaries, and it was reported to regulate primordial follicle assembly through its effects on proliferating cell nuclear antigen expression (Zhang et al., 2014). *MIR654* was also studied as one of the miRNAs changing levels in developing human brain (Moreau et al., 2013).

Changes in the levels of *MIR376* family members have been observed in various pathologies. *MIR376B* was shown to be upregulated in the ischemic heart, while its down- regulation resulted in cardioprotection (Pan et al., 2012). In a rat model of brain ischemia/reperfusion, *MIR376B* was found to be upregulated after 6h ischemic pre-conditioning (Dharap and Vemuganti, 2010). *MIR654* was also studied in various cellular settings including facioscapulohumeral dystrophy patients (Dmitriev et al., 2013) and H1N1 influenza A viral infection (Song et al., 2010); furthermore, it was down-regulated in acute rejection after renal transplantation (Sui et al., 2008). As an indication of the role for the *MIR376* family in virus-host interactions, differential expression patterns of *MIR376A* and *MIR376B* were observed in HIV-1 positive peripheral blood mononuclear cells compared to non-infected controls (Duskova et al., 2013).

Interplay between cellular and viral miRNAs was reported in various contexts and competitive or collaborative binding to target mRNA sequences (MREs) was observed. For example, 3'UTRs of the MICB mRNA (a stress-induced ligand necessary for host cell recognition and destruction by natural killer cells) was found to contain overlapping MREs for both *MIR376A* and Kaposi's sarcoma-associated herpesvirus (KSHV) *miRNA, miR-K12-7* (Nachmani et al., 2010). Human cytomegalovirus (HCMV) *miR-UL112* was also shown to bind sequences close to the *MIR376A MRE*. Interestingly, combinatory expression of KSHV or HCMV miRNAs with *MIR376A* potentiated MICB target down-regulation, leading to an attenuation of MICB-related immune responses. In the light of these

data, cellular miRNAs including *MIR376A* might be used by parasites such as viruses in order to attenuate antiviral responses.

Role of *MIR376* family in cancer

Comparative studies analyzing miRNA expression in cancerous tissues and normal non-tumorous counterparts have revealed that cancer type-specific changes occur in miRNA expression patterns. *MIR376* family members are among those shown to be subject to changes in different cancer types (Table 1, Fig. 3).

Lung cancers

Members of the *MIR376* family were shown to be differentially regulated in lung cancer. Results of a miRNA microarray expression profiling revealed that *MIR376A* was among miRNAs that were overexpressed in murine lung cancers (Liu et al., 2010). Further analysis confirmed that the observed changes were valid in a set of human lung cancers with different pathologies. On the other hand, *MIR376B* was downregulated in a set of non-small cell lung carcinomas (Son et al., 2009). Additionally, *MIR654* was moderately downregulated following histone methyltransferase inhibition in a lung cancer cell line (Pang et al., 2014).

Breast cancers

Detection of circulating miRNAs in the blood might be used as a diagnostic tool in various diseases, including cancer. In order to analyze the role of circulating miRNAs in breast cancer, Cuk et al. carried out two separate studies in which they checked plasma obtained from breast cancer patients (Cuk et al., 2013a; 2013b). In the first study, analysis of miRNAs using TaqMan low-density arrays from plasma of early stage breast cancer patients revealed that, four miRNAs *MIR376C, MIR148B, MIR409-3p* and *MIR801* were upregulated compared to healthy control individuals (Cuk et al., 2013b).

In the second study, miRNA array-based screening was performed and in addition to previously reported

Table 1. Role of MIR376 family in cancer.

Cancer type	microRNA	Target of miRNA	State in cancer tissue	Circulating miRNA	References
Murine and human lung cancers	MIR376A	N.D.	Upregulated	N.D.	Liu et al., 2010
Non-Small Cell Lung Cancer	MIR376B	N.D.	Downregulated	N.D.	Son et al., 2009
Human lung cancer cells	MIR654	N.D.	Downregulated	N.D.	Pang et al., 2014
Breast cancer	MIR376C	N.D.	Upregulated	Plasma	Cuk et al., 2013a,b
Breast cancer	MIR376A	N.D.	Upregulated	Plasma	Cuk et al., 2013a,b
Breast cancer	MIR376B	N.D.	Deregulated	N.D.	Lowery et al., 2009
Metastatic prostate cell lines	MIR376A, MIR376C, MIR654	N.D.	Downregulated	N.D.	Formosa et al., 2014
Human prostate cancer cells	MIR654	Androgen receptor (AR)	Downregulated	N.D.	Ostling et al., 2011
Ovarian cancer cells	MIR376C	ALK7	Upregulated	N.D.	Ye et al., 2011
Ovarian cancer	MIR376A*	N.D.	Upregulated	N.D.	Kim et al., 2014
Ovarian cancer	MIR376A, MIR376B, MIR376C	N.D.	Downregulated	N.D.	Zhang et al., 2008
Endometrial serous adenocarcinoma	MIR654	N.D.	Upregulated	N.D.	Hiroki et al., 2010
Uterine Leiomyomas	MIR376B	N.D.	Downregulated	N.D.	Wang et al., 2007
Hepatocellular carcinoma tissues and cells	MIR376A	p85a (PIK3R1)	Downregulated	N.D.	Zheng et al., 2012
Hepatocellular carcinoma tissues and cells	MIR376A	HDAC9	Downregulated	N.D.	Zheng et al., 2015
Pancreatic ductal adenocarcinoma	MIR376A	N.D.	Upregulated	N.D.	Lee et al., 2007
Pancreatic cancer cell lines- Panc1	MIR376 precursor	N.D.	Upregulated	N.D.	Jiang et al., 2005
Osteosarcoma cells	MIR376C	N.D.	Downregulated	N.D.	Duan et al., 2011
Osteosarcoma cells	MIR376C	TGFα	Downregulated	N.D.	Jin et al., 2013
Rapidly proliferating osteosarcoma cell lines	MIR376A, MIR376C	N.D.	Upregulated	N.D.	Lauvrak et al., 2013
Chondrosarcomas	MIR376A, MIR376B, MIR376C, MIR376A*	N.D.	Downregulated	N.D.	Yoshitaka et al., 2013
Pleomorphic adenomas of the salivary gland	MIR376A	N.D.	Upregulated	N.D.	Zhang et al., 2009
Oral squamous cell carcinoma	MIR376C	N.D.	Downregulated	N.D.	Kozaki et al., 2008
Esophageal Cancer	MIR376A	N.D.	Upregulated	N.D.	Zhao et al., 2013
Gastric cancer	MIR376C	N.D.	Upregulated	Serum	Song et al., 2012
Human bile duct carcinoma	MIR376C	GRB2	Downregulated	N.D.	Iwaki et al., 2013
Colorectal cancer stromal tissue	MIR376A	N.D.	Upregulated	N.D.	Nishida et al., 2012
Colorectal tumors	MIR376A	N.D.	Downregulated	N.D.	Mo et al., 2014
Acute myeloid leukemia	MIR376C	N.D.	Upregulated	N.D.	Dixon-McIver et al., 2008
Acute myeloid leukemia	MIR376C	N.D.	Deregulated	N.D.	Li et al., 2008
Clear cell renal carcinoma	MIR376B	N.D.	Downregulated	N.D.	Nakada et al., 2008
Melanoma	MIR376A, MIR376C	IGFR1	Downregulated	N.D.	Zehavi at al., 2012
Glioblastoma	MIR376A, MIR376B, MIR376C	HSV-TK	RNA editing	N.D.	Skalsky et al., 2011
High grade gliomas	MIR376A*	RAP2A /AMFR	RNA editing	N.D.	Choudhury et al., 2012
VERO cells	MIR376A MIR376B MIR376C	N.D.	Upregulated	N.D.	Teferedegne et al., 2010

miRNAs, *MIR127-3p, MIR376A* and *MIR652* were also reported to be breast cancer detection markers (Cuk et al., 2013a). The combination of miRNA expression profiling with artificial neural network analysis allowed Lowery et al. to predict ER, PR and HER2/neu status of breast cancers with a high accuracy (Lowery et al., 2009). Interestingly, breast tumors positive for HER2/neu could be revealed using the miRNA set *MIR376B, MIR181C, MIR320C* and *MIR520D*, indicating that *MIR376* has potential diagnostic value for breast cancer detection.

Prostate cancers

Although widespread use of detection techniques including prostate-specific antigen (PSA) analysis has improved prostate cancer detection rates, discovery of novel cancer markers that will allow earlier cancer detection, prediction of treatment responses and prognosis are necessary. Therefore, expression patterns of a set of 750 miRNAs in prostate cancer cells were analyzed by Formosa et al., and the screen resulted in the discovery of several tumor-related miRNAs (Formosa et

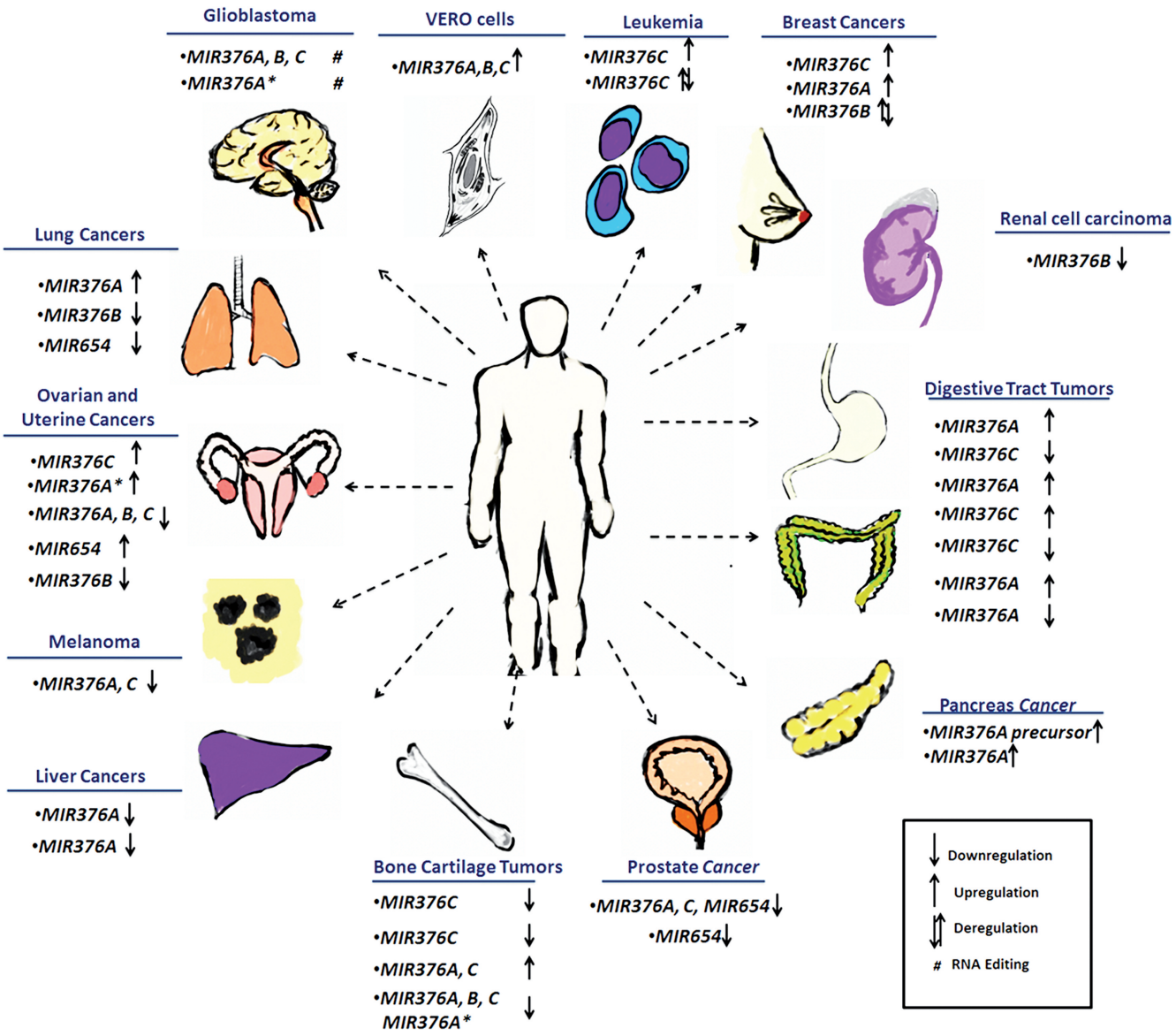


Fig 3. MIR376 family in cancer. Expression analysis of MIR376 family members in different cancer types that were shown in the literature. Downregulation, upregulation, deregulation and editing events are marked using the indicated symbols (upward or downward facing arrows or a hashtag).

al., 2014). Strikingly, in metastatic prostate cancer cells, but not in normal prostate epithelial cells, a consistent down-regulation was observed in the levels of miRNAs from the 14q32.31 locus, including *MIR376* family members *MIR376C, MIR376A* and *MIR654*. Ectopic expression of these miRNAs resulted in suppression of proliferation, migration and invasion of prostate cancer cells. In another study, a panel of human prostate cancer cells and 1129 miRNAs were used to perform a gain-of-function screen (Ostling et al., 2011). Here, changes in androgen receptor (AR) protein levels were used as a read-out.

Among 71 miRNAs discovered from the screen, 13 of them , including *MIR654*, were shown to directly affect the AR mRNA 3'UTR region. Importantly, miRNAs that were down-regulating AR could attenuate hormone-induced proliferation of prostate cancer cells, establishing the fact that miRNAs, including *MIR654*, are important regulators of androgen-dependent cancer growth.

Ovarian and uterine cancers

At least three different studies reported deregulation of *MIR376* family members in ovarian cancer. Ye et al. demonstrated that *MIR376C* promoted survival and proliferation of ovarian cancer cells through suppression of proapoptotic ALK7 (activing receptor-like kinase) protein and its ligand Nodal (Ye et al., 2011). In another study, a screen of microRNA expression in Taxol-resistant ovarian cells showed that 82 miRNAs were deregulated in ovarian cancer cells compared to normal ova (Kim et al., 2014). *MIR376A** was found among overexpressed miRNAs, introducing it as predictor of Taxol resistance in ovarian cancer. Zhang et al. also carried out an analysis of miRNAs that were deregulated in ovarian cancer. They discovered that down regulation of miRNAs from the Dlk1-Gtl2 locus was associated with increased tumor proliferation and shorter patient survival. The study attributed a tumor suppressor role for eight miRNAs located to chromosome 14, including *MIR376* family members *MIR376A, MIR376B and MIR376C* (Zhang et al., 2008).

Changes in *MIR376* family expression were also observed in uterine cancers. *MIR654* levels were upregulated in endometrial serous adenocarcinomas and dysregulation of miRNAs, including *MIR654*, was associated with poor prognosis in patients (Hiroki et al., 2010). Additionally, *MIR376B* was reported among down-regulated *miRNAs* in uterine leiomyomas (Wang et al., 2007).

Liver cancers

MiRNAs seem to play a role in liver cancers as well. Zheng et al. found out that *MIR376A* was frequently down-regulated in the early phases of liver regeneration. In line with this, miRNA overexpression suppressed cell proliferation and induced apoptosis in hepatocellular carcinoma (HCC) cells; *MIR376A* overexpression also slowed down HCC growth in nude mice xenografts. *PIK3R1* (p85a) was the direct target for the microRNA in this context, and an inverse correlation was revealed between the expression of *PIK3R1* and *MIR376A*. These results indicate a tumor suppressive role for *MIR376A*, and its down regulation in HCC might contribute to cancer development (Zheng et al., 2012). The same group recently published a report about an epigenetic mechanism behind the deregulation of *MIR376A* in HCC (Zheng et al., 2015). They observed that histone deacetylase (HDAC) inhibitors increased *MIR376A* levels. In fact, the Dlk1-Dio3 region was previously described as an imprinted region through DNA methylation and histone acetylation (Stadtfeld et al., 2010; McMurray and Schmidt, 2012). HDAC9, which targets the acetyl-histone H3K18, was identified as a direct target of *MIR376A*, and knockdown of HDAC9 led to the up regulation of *MIR376A, MIR376B* and *MIR376C* in addition to *MIR494* (Zheng et al., 2015). Moreover, HDAC9 expression was increased in HCC tissues. All these data indicate that a feedback loop regulates *MIR376A* levels in HCC, in which deacetylation of H3K18 by HDAC9 decreases *MIR376A* expression levels during cancer proliferation.

Pancreatic cancer

The level of *MIR376* family members in pancreatic cancer has been studied in pancreatic ductal adenocarcinoma and *MIR376A* was found to be upregulated (Lee et al., 2007). Another study using pancreatic cancer cell lines showed that expression of the *MIR376A* precursor was the highest in Panc-1 among all studied cell lines (Jiang et al., 2005).

Bone and cartilage tumors

The role of *MIR376* family members, especially *MIR376C*, in osteosarcomas has been investigated in various studies. In a study by Duan et al., miRNA expression profiles were analyzed, and three miRNAs including *MIR376C* (others being *MIR199A-3p* and *MIR127-3p*) were found to exhibit lower expression levels in osteosarcoma cell lines, while *MIR151-3p* and *MIR191* levels were increased (Duan et al., 2011). Interestingly, overexpression of one of the miRNAs, namely *MIR199-3P*, decreased cellular growth and migration, implicating a role for miRNA dysregulation in osteosarcoma proliferation and metastasis. In line with these results, Jin et al. showed that overexpression of *MIR376C* in osteosarcoma cells led to a decreased expression of TGF alpha (TGFα) through direct targeting of the oncogene, and resulted in an impairment of growth, migration and invasion capacity (Jin et al., 2013). In contrast, Lauvrak et al. showed that *MIR376A* and *MIR376C* levels were upregulated in rapidly proliferating osteosarcoma cell lines compared to slower proliferating counterparts (Lauvrak et al., 2013). Further

studies including analysis of samples from tumors at different stages are required to elucidate the role of *MIR376* member expression in osteosarcomas and to reconcile these conflicting results.

Tumors of cartilage were also analyzed. A large screen involving miRNA expression profiling in chondrosarcomas revealed that, several miRNAs from the *MIR376* family, namely *MIR376A, MIR376A*, MIR376B* and *MIR376C* were significantly down-regulated in chondrosarcomas compared to non-tumorous articular chondrocytes (Yoshitaka et al., 2013).

Digestive tract tumors

MIR376 family members were reported to be deregulated in several digestive tract tumors.

MIR376A levels were upregulated in pleomorphic adenomas of the salivary gland, a common type of tumor in this tissue (Zhang et al., 2009). In contrast, *MIR376C* was down regulated in oral squamous cell carcinoma cell lines (Kozaki et al., 2008). Screening of microRNAs in esophageal cancers with lymph node metastasis revealed that *MIR376A* levels were upregulated in tumors compared to control non-tumoral tissues (Zhao et al., 2013). In gastric cancer patients, serum *MIR376C* levels were upregulated, and screens using the miRNA allowed distinction between gastric cancer from superficial gastritis (SG) and mild chronic atrophic gastritis (CAG) (Song et al., 2012). The same miRNA was repressed in the HuCCT1 human bile duct carcinoma cell line, and its direct target GRB2 was shown to be important in EGF-dependent cell migration (Iwaki et al., 2013). In a study using microarray analysis and qPCR, *MIR376A* was found to be upregulated in colorectal cancer stroma compared to control tissues (Nishida et al., 2012). Another study on colorectal cancer showed that *MIR376A* was expressed at lower levels in tumors compared to adjacent normal tissues, but overexpression of the miRNA in tumors correlated with higher lymph node metastasis and shorter patient survival (Mo et al., 2014).

Leukemia

Two different studies showed that *MIR376C* levels were deregulated in acute leukemia. In the first study, expression profiles of 157 mature miRNAs from 100 AML (acute myeloid leukemia) patient samples were analyzed (Dixon-McIver et al., 2008). AML bearing a t (15;17) translocation had a distinctive signature involving upregulation of a subset of miRNAs including *MIR368* (*MIR376C*).

In the other study, miRNA expression analysis in 52 acute myeloid leukemia (AML) samples revealed that a set of miRNAs including *MIR376C* could distinguish t (15;17) positive AML from other leukemia subtypes (Li et al., 2008). These results indicate that differential miRNA expression patterns might serve diagnostic purposes and together with other selected miRNAs, *MIR376C* could be used to differentiate AMLs with specific translocations.

Other cancer types

Cancers from other tissues have also been analyzed. *MIR376B* was reported to be down regulated in clear cell carcinomas of kidneys (Nakada et al., 2008). Similarly, *MIR376A* and *MIR376C* were downregulated in melanomas, leading to the overexpression of their specific target IGFR1, and possibly contributing to increased tumorigenesis and metastasis (Zehavi et al., 2012). In glioblastomas, editing of *MIR376* family members, *MIR376A, MIR376B* and *MIR376C* was observed (Skalsky and Cullen, 2011). Yet, unedited *MIR376** transcripts were found in high-grade gliomas, and this native miRNA form was associated with invasive tumor spread (Choudhury et al., 2012). Indeed, unedited *MIR376** affected cell migration and invasion *in vitro* and *in vivo* in xenograft mice models, while the edited form suppressed these changes. Similar results were obtained with *MIR376A* in neoplastically transformed Vero monkey kidney cells. When *MIR376A* was overexpressed alone or as part of an expression cassette containing *MIR376A, MIR376B* and *MIR376C*, cell migration and matrigel invasion of Vero monkey cells were reported to increase (Teferedegne et al., 2010).

The above-mentioned studies indicate a correlation between the expression levels of *MIR376* family and malignant transformation and invasiveness.

The *MIR376* family and chemotherapy

Accumulating literature points to a significant role for MIR376 family in determining sensitivity of tumor cells to insults including chemotherapy. For example, in ovarian cancer cells, *MIR376C* overexpression conferred cellular resistance to anticancer drugs cisplatin and carboplatin, introducing the miRNA as a determinant factor for chemoresistance (Ye et al., 2011). In line with these results, *MIR376A** was found among over-expressed miRNAs in taxol-resistant ovarian cancers compared to those sensitive to the drug (Kim et al., 2014). Along the same line, *MIR376A* was indicated as a miRNA that confers resistance to chemotherapy in esophageal cancer cells. In fact, the miRNA was downregulated following treatment with the proton pump inhibitor esomeprazole, a drug that was shown to potentiate the cytotoxic effects of cisplatin and 5-Fluorouracil in esophageal cancer cells (Lindner et al., 2014). Moreover, low circulating *MIR376A** levels were suggested among predictors of non-small-cell lung cancer patient overall survival following treatment with a combination chemotherapy regimen (bevacizumab and erlotinib followed by platinum) (Joerger et al., 2014). In retinoblastoma cells, arsenic trioxide treatment led to a down regulation of *MIR376A* expression, and over-expression of the miRNA using mimics significantly

blocked cell death induced by the drug (Zhang et al., 2013). In another study, *MIR376A* was shown to sensitize cervix and liver cancer cells to death induced by DNA damaging agents such as radiation, camptothecin or etoposide (Sheng et al., 2013). In fact, in fibroblasts *MIR376A** was previously reported to induce double-strand DNA breaks and accumulation of reactive oxygen species (Faraonio et al., 2012), while *MIR376C* levels were down-regulated in response to DNA damaging UV light exposure (Guo et al., 2009).

All these data indicate that cellular levels of the *MIR376* family members might be used as an indicator of the responsiveness of cancer cells to chemotherapy regimens. A cancer type-specific approach might open the way for the use of these miRNAs as cancer biomarkers. Evaluation of *MIR376* family members might potentially lead to modification of treatment strategies to achieve a more effective cancer treatment.

MIR376 family, autophagy and cancer

The autophagy-cancer connection is a relatively new area of research and microRNAs including the *MIR376* family have been implicated in autophagy regulation. Therefore, in this section, we will briefly introduce the autophagy-cancer connection and discuss the role of *MIR376* members in autophagy regulation in cancer cells.

Macroautophagy (autophagy hereafter) is a basic cellular response mechanism to stress. During autophagy, double or multimembrane vesicles engulf portions of cytoplasm, cytoplasmic proteins and organelles such as mitochondria, and deliver the cargo to the lysosomal/vacuolar system (Kim et al., 2002). Degradation of the cargo is followed by recycling of building blocks back to cytosol for reuse by the cell. Being an evolutionarily conserved mechanism in organisms ranging from plants (Kuzuoglu-Ozturk et al., 2012) to man (Gozuacik and Kimchi, 2004), autophagy is observed at basal levels in all cell types allowing basic protein degradation and organelle turnover. But under stress conditions such as starvation or exposure to toxins, autophagy is rapidly upregulated allowing stress resistance via sustaining the building blocks for cellular biosynthetic pathways and regulating homeostasis (Kim et al., 2002).

Studies in the last decade have showed that autophagy plays a critical role in several human diseases including cancer (Mizushima et al., 2002; Cuervo, 2004). Autophagy abnormalities were observed during cancer formation and progression.

A context-dependent link exists between autophagy and cancer: in the early stages of carcinogenesis and cancer initiation, a tumor-suppressor role has been attributed to autophagy. Indeed, autophagy is responsible for the elimination of organelles such as mitochondria, control of reactive oxygen release and protection from DNA damage, preserving genomic stability (Mathew et al., 2009). However, in established tumors and especially in solid tumors with oncogenic K-RAS pathway activation, autophagy was shown to contribute to cancer growth, allowing resistance to unfavorable conditions that occur in some tumor regions due to inefficient tumor vascularization (i.e. lack or low levels of nutrient supply and oxygen in spite of high metabolic requirements) (Levine et al., 2007). Moreover, autophagy seems to confer resistance to anoikis, protecting cells from death following detachment from the basal lamina (Fung et al., 2008). This facilitates cancer cell escape from primary tumors, leading to cancer invasion and spread. Autophagy activation capacity of cells also correlated with cancer cell dormancy and chemotherapy resistance (Gewirtz, 2009). In line with these data, some proteins involved in autophagy regulation have been reported to have roles as tumor suppressors or oncogenes (Gozuacik and Kimchi, 2004; Morselli et al., 2009).

The *MIR376* family was shown to regulate autophagy in cancer cells. In order to discover miRNAs involved in autophagy regulation, we performed an unbiased screen in MCF7 breast cancer cells using the autophagy marker GFP-LC3 (Korkmaz et al., 2012). LC3 is a protein that functions in the elongation and vesicle completion stages of autophagy. Two ubiquitylation-like reactions result in covalent binding of the LC3 (MAP1LC3 in mammals or ATG8 in yeast) protein to a lipid molecule, namely phosphatidyl ethanolamine.

While the free cytosolic form of the GFP fusion of LC3 (GFP-LC3) shows a diffuse intracellular localization, recruitment of LC3 to lipid molecules that associate with autophagic vesicles results in a cytosolic punctate staining. This test is commonly used rapidly to detect autophagy activation in cells. Using the GFP-LC3 test, we identified miRNAs that blocked starvation-induced autophagy. Among them were two *MIR376* family members: *MIR376A* and *MIR376B*. Further analysis confirmed the inhibitory effect of the miRNAs on MCF7 breast cancer and Huh7 hepatocellular carcinoma cell autophagy that was activated by starvation or mTOR-inhibition. *MIR376* family members exerted these effects through their direct binding to the 3' UTR sequences of the messenger RNAs of two key autophagy proteins, BECN1 (Beclin 1) and ATG4C (Korkmaz et al., 2012, 2013). Indeed, both mRNA and protein levels of BECN1 and ATG4C were decreased following overexpression of the miRNAs; while blockage of endogenous miRNAs using antagomirs (anti-miRNAs) had the opposite effect. Interestingly, endogenous miRNA levels were rapidly increased following exposure to autophagy-activating signals, and *MIR376A*- or *MIR376B*-specific antagomirs led to a "hyperactivation of autophagy" ((Korkmaz et al., 2012, 2013) and Tekirdag and Gozuacik unpublished results).

These results are consistent with a gas-and-brake mechanism operating in cancer cells. Stress signals trigger autophagy activation in cells (the gas), but meanwhile, the same signals also stimulate expression of autophagy inhibitory miRNAs (the brake). Hyper-

activation of autophagy might be harmful to cells because it may result in an excessive elimination of mitochondria, other vital organelles and survival-related proteins. The gas-and-brake mechanism relies on *MIR376* family members, and it serves to limit the amplitude of the autophagy response, preventing hyperactivation of autophagy beyond physiological needs and protecting cells from damage.

As mentioned in previous sections, deregulation of *MIR376* family members was observed in various cancer types. Since autophagy activation is vital for some rapidly growing solid tumors, *MIR376* family levels may be a critical factor for cancer cell survival and growth.

In these rapidly growing tumors, normal or lower expression levels of *MIR376* family members are expected, allowing efficient autophagy activation that meets the needs of cancer cells. On the other hand, autophagy defects were associated with accumulation of abnormal mitochondria, aggregate proteins and oxidative stress, leading to genomic instability and higher mutation rates (Mathew et al., 2009). Therefore, *MIR376* family overexpression that was observed in some tumor types might be associated with polyclonal expansion, and emergence of aggressive and/or drug resistant cancer phenotypes. To complicate the picture, the literature on the miRNA-autophagy connection is expanding, and the number of miRNAs that might stimulate or inhibit autophagy is increasing. Although further independent studies are required to reveal the relevance and importance of *MIR376* family-regulated autophagy in individual tumor types, our preliminary *in vitro* and *in vivo* studies indicate that autophagy plays a central role in the cancer-related effects of the *MIR376* family (Tekirdag et al. manuscript in preparation).

Conclusions and future perspectives

In recent years, microRNAs were studied extensively as endogenous regulators of gene expression and as critical coordinators of various biological phenomena. These potent small RNA molecules have been shown to play various physiological roles, and not surprisingly, their dysregulation is associated with a number of pathological conditions.

Members of the *MIR376* family were studied by several independent groups and in different contexts. A wide range of tissues was reported to express *MIR376* family members indicating that these are not tissue-restricted miRNAs. Moreover, a developmental expression regulation pattern that was observed for the *MIR376* family implicates a role for these miRNAs in embryonic development control. Interestingly, several studies point to a role for the *MIR376* family in ovarian development and maturation. Brain development is another interesting biological event where *MIR376* family members seem to be active. The fact that several edited forms of *MIR376* family were observed in different brain areas and in different developmental stages confirms that they are tightly regulated in the brain.

Expression profiling studies and other analyses have revealed that *MIR376* family members might act as tumor suppressors or oncogenes, underlining disease- and/or context-dependency of their functions. As summarized above, dysregulation of *MIR376* family members was observed in various carcinomas and sarcomas, solid tumors and leukemia. Importantly, in some studies, expression levels of *MIR376* family members correlated with tumor grade, invasiveness, metastasis and/or chemotherapy responsiveness. Therefore, it is clear that miRNAs belonging to this family have huge potential as novel cancer biomarkers. Advances in nanotechnology open the way for the usage of small RNA molecules for cancer treatment purposes.

For example, mimics or antagomirs derived from microRNAs, including *MIR10B, MIR34A, MIR107* and *MIR155* were successfully used as anticancer drugs in several preclinical studies (Gozuacik et al., 2014). In this context, combination of non-liposomal particles with small RNAs were found to be safe and feasible in non-human primates and human, and clinical studies gave hope about their potential use in cancer treatment (Heidel et al., 2007; Davis et al., 2010; Tabernero et al., 2013). In fact, selected nanoparticle carriers might potentially be targeted into tumors and concentrate in the near-tumor environment (Gozuacik et al., 2014). In the light of these promising studies, and literature-based facts about connections between *MIR376* family members and various cancer types, exploitation of *MIR376* family as gene therapy targets or tools might be possible in the near future.

MiRNA-based treatments might provide novel and somewhat futuristic cancer treatment strategies when combined with tumor-targeting nanoparticles. Combinations of conventional cancer drugs with single or miultiple miRNA regimens might offer alternatives to toxic and non-targeted classical chemotherapy drugs, minimizing serious drug-related side-effects. Further studies will reveal the potential of *MIR376* family members as cancer drugs or drug targets.

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