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Consequences of genetic variants in miRNA genes
Magdalena Machowska, Paulina Galka-Marciniak, Piotr Kozlowski

Department of Molecular Genetics, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Noskowski 12/14, 61-704 Poznan, Poland

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ABSTRACT

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* Corresponding author.
E-mail addresses: mmachowska@ibch.poznan.pl (M. Machowska), pgalka@ibch.poznan.pl (P. Galka-Marciniak), kozlowp@ibch.poznan.pl (P. Kozlowski).

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1. Introduction

MicroRNAs (miRNAs) are small (~21–23 nt) single-stranded noncoding RNAs whose canonical function is posttranscriptional regulation, usually downregulation, of the expression of their target genes. The specificity of target recognition is conditioned by the complementary interaction of miRNAs with their target sites, usually located in the 3'-untranslated region (3' UTR) of the targeted mRNAs. There are approximately 1900 and 567 miRNA precursors/genes annotated for humans in the most reputable miRNA databases, i.e., miRNABase v.22.1 [1] and miRGenDB v.2.1, [2,3], respectively. miRNA genes may be located within protein-coding genes or constitute independent transcriptional units (often annotated as miRNA host genes) and may encode either one or multiple miRNAs arranged in so-called miRNA clusters [4]. miRNA biogenesis is a multistep process described in detail elsewhere [5,6]. Briefly, most miRNA genes are transcribed by RNA polymerase II. The most crucial part of the miRNA primary transcript (pri-miRNA) is a characteristic hairpin structure that upon cleavage by the microprocessor complex, in which the most important part is RNase DROSHA accompanied by DGCR8 and several other proteins, is released to the nucleoplasm as a secondary miRNA precursor (pre-miRNA). The pre-miRNA is exported to the cytoplasm, where it is further processed by the miRNA-induced silencing complex (miRISC) loading complex (RLC). The main component of the RLC is RNase DICER1, which cuts off the apical loop of the hairpin-shaped pre-miRNA, generating an ~22-bp-long miRNA duplex, usually with 2 nucleotide-long 3'-end overhangs. Within the miRISC, the miRNA duplex is unwound, separating miRNA strands. One of the strands becomes the mature miRNA (miR-5p or miR-3p) and functions as a guide strand to recognize and silence target mRNAs, a process that is assisted by AGO and TNR6A proteins. It should be noted that the biogenesis of some miRNAs may be further regulated by different RNA-binding proteins and may be conducted by other alternative pathways [7,8].

miRNAs regulate many important biological processes, e.g., the development, differentiation, and maintenance of homeostasis [9]. Many miRNAs have been identified to be upregulated or downregulated in different physiological and pathological conditions (diseases), and the role of many miRNAs in different human diseases, including various cancers, has been convincingly demonstrated [10].

Nonetheless, little attention has been given to the genetic variations in miRNA genes and their potential consequences for the functioning of miRNA genes, aberrations of miRNA-regulated processes, and human conditions, including diseases. Except for mutations in miRNA seed regions, which are crucial for target recognition, there is little knowledge or guidelines on how to interpret mutations located in other parts of miRNA genes. Are they all neutral variants or potentially deleterious mutations? In this review, we present the representative examples of single nucleotide polymorphisms (SNPs), germline mutations in Mendelian diseases, and cancer somatic mutations identified thus far in miRNA genes to discuss the potential consequences of genetic variants in these genes. We focused on the structural and functional consequences of genetic variants located in the different parts of miRNA genes, and their effect on miRNA genes expression, miRNA precursor structure, miRNA biogenesis, level of mature miRNA strands, and recognition of miRNA targets. We also discuss the implications of miRNA variants for human diseases.

2. Potential effects of genetic variants on miRNA biogenesis and function

The particular sequence motifs and structural conformation of miRNA precursors are crucial for the proper biogenesis of miRNAs (Fig. 1A). Any aberrations caused by mutations or SNPs may lead to alterations at a particular step of miRNA biogenesis, resulting in changes in the miRNA level, precision of miRNA processing, 5p/3p miRNA balance, and specificity of miRNA target recognition (Fig. 1).

The primary effect of each mutation in sequences coding for miRNA precursors, regardless of the subregion of the precursor in which the mutation occurs, is altered precursor structure and stability. This effect may be minor and may not change the general geometry of the precursor structure or be more profound and completely rearrange/destroy the structure. The effect of a mutation on the structure, among others, depends on the type of mutation, its localization, and the thermodynamic properties (stability) of the precursor. Generally, it may be expected that mutations/structural rearrangements in or in the direct vicinity of a miRNA hairpin structure (pre-miRNA) may have a more substantial effect on miRNA biogenesis and functioning of miRNA genes than mutations in other parts of the precursor (pri-miRNA) [11]. The mutations inducing larger structural rearrangements can make the precursor unrecognizable to the miRNA biogenesis enzymes and thus may stop miRNA biogenesis (Fig. 1B). Minor structural changes, such as new or removed bulges, internal loops, or other rearrangements inducing local changes in structure stability, may affect the precision or efficiency of miRNA precursor processing, mature miRNA selection and 5p/3p strand balance (Fig. 1B) [12]. Regardless of the impact on the structure, mutations in miRNA genes may disrupt or create new protein binding sites of RNA-binding proteins (RBPs), thus affecting the regulation of proper miRNA processing (Fig. 1C) [13]. Mutations in DROSHA or DICER1 cleavage sites may block or affect the precision of cleavage (Fig. 1D), which in turn may affect the balance between isomiRs or lead to the generation of new isomiRs [14–16]. Furthermore, variants in the miRNA duplex may change its stability, affecting the preferences of mature strand selection, and 5p/3p strand balance (Fig. 1E). The most obvious effects of mutations on miRNA functionality are due to mutations that occur in seed regions. A seed mutation in most cases will preclude proper recognition and silencing of its targets, but it may also lead to the recognition of novel targets (Fig. 1F) [17].

In addition to mutations in miRNA precursors, mutations in miRNA gene promoters and other regulatory sequences may affect the function of miRNA genes. Genetic variants in promoters may destroy, modify, or create new transcription factor-binding sites and thus may affect transcription efficiency and miRNA levels (Fig. 1G).

Mutations in the 3'UTRs of target genes and in genes that play a role in miRNAs biogenesis may also influence miRNAs functionality and biogenesis. However, in this review, we did not focus on these aspects, as they are well described in other publications [18–21]. Still, other mechanisms that may have an impact on the expression of miRNAs genes and function of miRNAs are epigenetic regulation of gene expression by promoters methylation [22,23], methylation of miRNA-coding genomic regions [24], or histones modifications [25], as well as posttranscriptional modifications as A-to-I RNA editing [26,27] or methylation of adenosine, guanosine or cytosine in miRNAs [28], but also these aspects are not the subject of this review.

Although most alterations in miRNA genes most likely act as loss-of-function mutations, some of these alterations may also be gain-of-function mutations. Such mutations could generate new miRNAs that recognize and downregulate new targets or create new competing endogenous RNAs (ceRNAs). New miRNAs may result from mutations in seed regions, mutations generating new isomiRs, or the preferential selection of a previously unused strand. Gain-of-function mutations may also result from the creation of new protein binding sites either in the promoter or in the miRNA precursor.
The examples presented above include those that may be directly deduced from the current knowledge about miRNA biology and certainly do not cover all the possibilities of how mutations may affect the functioning of the miRNA genes.

3. Seminal analyses of genetic variants in miRNA genes

The first global analysis of SNPs in miRNA genes showed that the density of SNPs within the precursor (hairpin-forming) sequence of miRNA genes is significantly lower than that in ~300-nucleotide-long flanking regions [29]. This indicates that sequences coding for pre-miRNAs are crucial parts of miRNA genes in which genetic variation are not well-tolerated and confirms the functional importance and conservativeness of these sequences. This seminal result was subsequently confirmed in several later studies, which took advantage of a growing number of annotated SNPs and human miRNAs [30–33]. In later studies that analyzed the SNP density in the subregions of miRNA precursors, it was...
| miRNA gene | HGVS; dbSNP ID | variant type | main mRNA region (position)<sup>b</sup> | free energy [kcal/mol]<sup>*</sup> | pri-miR level | pre-miR level | 5p-miR level | 3p-miR level | implicated disease | references |
|------------|----------------|--------------|---------------------------------|---------------------------------|--------------|--------------|--------------|--------------|------------------|-----------|
| MIR15A/MIR16-1 cluster | | wild-type | | | | | | | | |
| MIR30C2 | n.77C>A; reference allele n.16C>A; rs1296517452 wild-type | germline mut | 5p flank | -31.80 | -32.3 | -29.3 | - | - | - | CLL (cancer predisposition) Calin 2005; Allegra 2011 |
| MIR96 | n.15G>A | germline mut | 5p seed (4th nt) | -48.40 | -41.30 | - | - | no change | no change | NSHL (Mendelian) Mencia 2009; Solda 2012 |
| MIR96 | n.14C>A | germline mut | 5p seed (5th nt) | -41.80 | - | - | - | - | - | NSHL (Mendelian) Mencia 2009; Solda 2012 |
| MIR96 | n.57T>C | compensatory | 3p seed (5th nt), miR-5p (15th nt) | -49.80 | - | - | - | - | - | AML, other blood cancers Marshall 2020; Kwanhian 2012 |
| MIR125A | reference allele n.22G>T; rs12975333 SNP | germline mut | 5p seed (7th nt) | -46.10 | -40.00 | | | | | Duan 2007 |
| MIR140 | n.24A>G | germline mut | 5p seed (1st nt) | -44.30 | -52.00 | | | | | skeletal dysplasia (Mendelian) Grigelioniene 2019 |
| MIR142 | n.55A>G | somatic mut | 3p seed (3rd nt) | -44.00 | - | - | | | | AML, other blood cancers Trissal 2018; Marshall 2020 |
| MIR146A | SNP | germline mut | 3p seed (3rd nt) | -40.30 | - | | | | | AML, other blood cancers Trissal 2018; Marshall 2020 |
| MIR184 | n.57C>T | germline mut | 3p seed (4th nt) | -35.50 | -38.50 | - | - | - | - | keratoconus, EDICT (Mendelian) Hudges 2011, Iloff 2012 |
| MIR204 | n.37C>T | germline mut | 5p flank | -33.40 | -34.70 | - | - | - | - | keratoconus (Mendelian) Lechter 2013 |
| MIR499A | n.73A>G; rs3746444 | germline mut | 5p seed (3rd nt) | -48.40 | -45.00 | | | | | keratoconus (Mendelian) Lechter 2013 |
| MIR502 | n.13C>G; rs782404826 SNP | germline mut | 5p flank | -59.00 | -39.20 | -39.50 | - | - | - | keratoconus, EDICT (Mendelian) Lechter 2013 |
| MIR510 | n.6G>A; rs1548999778 reference allele | germline mut | 5p flank | -39.20 | -39.50 | - | - | - | - | keratoconus, EDICT (Mendelian) Lechter 2013 |
| MIR510 | n.48T>C | SNP | 3p seed (3rd nt) | -35.50 | -39.20 | - | | | | |
| MIR5890 | n.69G>C; rs158166791 SNP | miR-3p (21st nt) | -32.20 | - | | | | | | |
| MIR822B | SNP | germline mut | 5p seed (4th nt) | -42.70 | - | | | | | |
| MIR934 | SNP | SNP | 3p seed (15th nt) | -38.10 | - | | | | | |

<sup>a</sup>Associated with different cardiovascular diseases and cancers Ding 2018
<sup>b</sup>Associated with PTC and other cancers, autoimmune and infectious diseases Jazdzewski 2008/Shen 2008; Lofgren 2012; Zhang 2014

References:
- Calin 2005; Allegra 2011
- Iwai 2005
- Mencia 2009; Solda 2012
- Trissal 2018; Marshall 2020
- Hudges 2011, Iloff 2012
- Lechter 2013
- Solda 2012
- Mencia 2009
- Trissal 2018; Marshall 2020
- Jazdzewski 2008/Shen 2008; Lofgren 2012; Zhang 2014
- Ding 2018
- Sun, 2009
shown that SNP density is almost equally decreased in the seed, loop, and miRNA duplex sequences. This finding indicates that all parts of pre-miRNA sequences (not only seeds) are similarly sensitive to genetic variants and suggests the substantial functional potential of all mutations in pre-miRNA sequences [31–33]. A similar conclusion about the conservative character of miRNA genes can also be drawn from the observation that the density of common copy number variants (CNVs) is significantly decreased in the regions of miRNA genes in comparison to the whole genome [30].

Despite the decreased density of SNPs in sequences coding for miRNA precursors, there are still numerous SNPs identified in these regions. The first SNPs in miRNA precursor-coding sequences were identified experimentally by sequencing 173 human pre-miRNA sequences (all known at the time of the analysis) in 96 samples from Japanese individuals [34]. The experiment allowed the identification of 10 SNPs located in 10 different pre-miRNA sequences. The identified SNPs were located in different subregions of pre-miRNAs, including mature miRNAs, passenger strands, apical loops, and directly adjacent (up to ∼10 nucleotides) flanking sequences. Among the identified SNPs was a substitution located in MIR30C2 in the 10th nucleotide of mature miR-30c-2-5p (n.16C>A; mutation annotation based on HGVS nomenclature, in reference to precursor transcript sequences from miRBase v.22.1) (Table 1). The mutation led to increased level of pre-miR-30c-2 and was predicted to affect the stability of miRNA:target pairing and proper target recognition.

Subsequent computational analysis of SNPs annotated in dbSNP revealed 12 SNPs located in 12 different pre-miRNAs (annotated in miRBase 2007). Among the identified SNPs was a G>T substitution (rs12975333; n.22G>T) in MIR125A, located at the 7th nucleotide of the miR-125a-5p seed sequence (Table 1). In addition to affecting target recognition and reducing the effectiveness of miRNA-mediated translational suppression, it was shown in vitro experiments that the alternative allele (T) introduces an additional mismatch to the precursor duplex and blocks precursor processing from pri-miRNA to pre-miRNA [35].

Another early study investigated the effects of 24 SNPs identified in schizophrenia and autism patients in miRNA genes linked to chromosome X [36]. The SNPs were located in different subregions of the miRNA precursors. Subsequent systematic in vitro analyses demonstrated that six of the SNPs which were analyzed in detail, regardless of their location in miRNA precursors, affect miRNA biogenesis. One of the SNPs detected in MIR510 (n.6G>A located in 5p flank region), resulted in an increased level of the mature miRNA, four SNPs, i.e., in MIR502 (n.13C>G located in 5p flank region), MIR510 (n.48T>C located in 3rd nt in 3p seed sequence), MIR890 (n.66G>C in 21st nt in 3p strand), and MIR892B (n.60T>C in 15th nt in 3p strand) resulted in a decreased level of the mature miRNAs, and one SNP in MIR934 (n.15T>G in 1st nt in 5p strand) resulted in altered DROSHA/DICER1 cleavage sites and the generation of novel miRNA (isomiR) (summarized in Table 1).

The abovementioned studies were the first to demonstrate the effects of genetic variants on miRNA biogenesis and the function of miRNA genes. However, as an increasing number of genetic variants in miRNA genes are detected, e.g., the latest genome-wide study identified 1994 SNPs (identified in the 1000 Genomes Project samples) in 1025 (out of 1904 annotated in miRBase) miRNA precursors [33], future functional analyses of such variants are to be expected.

4. SNPs in miRNA genes associated with various diseases

Due to the function of miRNAs in the regulation of many biological processes and role in human diseases, numerous studies have
been conducted to investigate the association between SNPs in miRNA genes and various human phenotypes and diseases, and numerous such associations have been reported [21,37,38]. However, it must be noted that most of these associations have been identified in small studies, were not replicated in independent cohorts, and are not significant at the genome-wide level; therefore, the results of such association studies should be interpreted with caution. Below, we describe several of the most convincing or most intensively investigated examples.

4.1. MIRLET7 family promoters

Let-7 was one of the first discovered microRNAs. It was originally detected in Caenorhabditis elegans [39] and was also found in metazoa [40]. In the human genome, there are 12 LET7 genes encoding 9 different mature miRNAs, which are detected at high levels during embryogenesis and in most tissues in adults [2,41]. Let-7 family members play an important role during development and differentiation and act as tumor suppressors (commonly downregulated) in many types of cancers. Their functions have been discussed in many articles, including [41,42].

There are two common SNPs in the promoters of the MIRLET7 genes. These SNPs are rs10877887 T>C in the promoter of MIRLET7I, with a minor allele frequency (MAF) of ∼40 %, and rs13293512 T>C in the promoter of the MIRLET7A1/MIRLET7F1/MIRLET7D cluster, with an MAF of 27 % [43], in which the minor alleles was suggested to decrease level of the corresponding miRNAs [44–46]. In silico analysis suggested that these SNPs may affect the affinity or binding sites for the predicted transcription factors myeloid zinc finger 1 (MZF1) and interferon regulatory factor 1 (IRF1) for rs10877887 and rs13293512, respectively. Moreover, rs10877887 is located in a CpG island, and thus, it may lead to the downregulation of MIRLET7 expression by epigenetic mechanisms [47]. Subsequent association studies showed that these SNPs are associated with the risk of numerous diseases, including different types of cancer. Both rs10877887 and rs13293512 were associated with a risk of intracranial aneurysm (IA) [48] and major depressive disorder (MDD) [48]. Moreover, the C allele of rs10877887 was associated with an increased risk of death in patients with hepatocellular carcinoma (HCC) [49] and an increased risk of death in patients with advanced colorectal cancer [50], but these results were not always conclusive or confirmed in independent studies [51,52].

The C allele of rs2910164 was also associated with other diseases, including higher susceptibility (risk) to multiple sclerosis in women [71] and leprosy [72] and lower risk of ankylosing spondylitis [73] and psoriasis [58].

4.3. MIR499A

Another SNP in the miRNA gene commonly associated with different diseases is rs3746444 (n.73A>G) in MIR499A, with an MAF in the global population of ∼20 % [43]. This SNP is located in the 3rd nucleotide of the miR-499a-3p seed sequence.

The main miRNA generated from MIR499A is miR-499a-5p. It plays a role in the maturation of cardiomyocytes and cardiac differentiation. It targets mostly genes involved in early cardiogenesis, and its level increases during this process [74,75]. In a normal heart, it exerts antiproliferative activity, and its level is often decreased in pathological conditions in the heart [76,77]. On the other hand, it was reported that miR-499a-5p has proapoptotic and antiproliferative activity in cancer cells and is considered as a tumor suppressor [78,79].

The G allele replaces the Watson-Crick base pair A:U with the wobble base pair G:U in the miRNA duplex and slightly destabilizes the structure of the miRNA precursor. In cells transfected with both allele variants, in cells with the G vs A allele, the level of the primary miRNA was increased, pre-miRNA and miR-499a-3p levels did not change, and miR-499a-5p levels were significantly decreased [80]. These findings indicate that the main effect of the G allele (although located in the 3p seed sequence) is a decrease of miR-499a-5p, which is the more important functional strand [78,80].

The G allele of rs3746444 was associated with increased susceptibility to inflammatory arthritis [81], a higher risk of asthma [82], diabetic polyneuropathy, cardiovascular autonomic neuropathy [83], and cardiovascular diseases but not with congenital heart disease and cerebrovascular diseases [84]. It was also associated with various cancer types, and the meta-analysis of data from 65 cancer association studies showed an association between the G allele and the increased global risk of cancer as well as the risk of individual cancers, such as cervical squamous cell carcinoma, hepatocellular carcinoma, lung cancer, and prostate cancer, but
was not associated with AML, breast, bladder, colorectal, esophageal, gastric, and oral squamous cell cancers [85]. It must be noted, however, that most of the individual studies included in the meta-analysis were of a very low statistical power; therefore, these results should be interpreted with caution.

5. Mutations in miRNA genes in Mendelian and familial diseases

There are hundreds of well-characterized Mendelian disorders. Almost all of these diseases are caused by mutations in protein-coding genes. However, for the last several years, an increasing number of such diseases caused by mutations in genes coding for non-coding RNAs, including microRNA-coding genes, have been reported. The summary of basic characterization of all mutations described below is shown in Table 1.

5.1. MIR96 and non-syndromic hearing loss

The first discovered Mendelian disease attributed to mutations in a miRNA gene was non-syndromic hearing loss (NSHL) [86], a heterogeneous hereditary disease with an overall prevalence ranging from approximately 1 per 1000 in newborns to greater than 3 per 1000 in adolescents [87]. More than 120 genes/loci are attributed to this disease [88]. One of the NSHL loci, DFNA50, was mapped on 7q32; however, despite sequencing of many candidate protein-coding genes in the region, no causative mutation has been found [89]. Only a detailed genetic analysis of MIR96, also present in the locus, allowed the detection of two mutations segregating with the disease in two large Spanish families and demonstrated the dominant character of the mutations [86]. MIR96 is expressed in the cochlear in inner and outer hair cells and plays a role during their differentiation [90,91]. It is also important in auditory hindbrain development [92].

The mutations found in the patients with NSHL are located at the 4th (n.13G>A) and 5th (n.14C>A) nucleotides of the seed sequence of the 5p mature miRNA (miR-96-5p). As both mutations were located in the seed sequence, it was assumed that the main effect of the mutations was the disruption of miRNA targets recognition. This effect of mutations was confirmed by the use of a luciferase reporter assay that showed the reduced silencing of the predicted wild-type miRNA targets by the mutant miRNAs. It was also shown that these two mutations result in mismatches (internal single nucleotide loops) in the miRNA duplex, which destabilize the precursor structure (Fig. 2A) and lead to a decrease in the level of miR-96-5p [86]. However, another mutation identified later in the Irish NSHL family was found in the opposite strand in the 6th nucleotide of the 3p seed region (n.57T>C). This mutation introduced a mismatch to the miRNA duplex, enlarging the existing internal loop and destabilizing the precursor structure (Fig. 2A). Subsequent functional analyses showed that the mutation result in decreased levels of both miR-96-5p and miR-96-3p but not the level of pre-miR-96. This finding implies that mutation-induced structural aberrations affect the precursor cleavage by Dicer1. To further investigate the effect of the mutation on the structure of the miRNA precursor, in the opposite strand of the mutation, the additional compensating mutation (n.23A>G) was introduced, restoring the Watson-Crick pairing with the mutation residue and the original structure of the precursor (Fig. 2A). The double mutant showed similar levels of miR-96-5p and miR-96-3p and a similar level of miR-96-5p targets downregulation as that observed in the wild-type [93]. As MIR96, along with MIR182 and MIR183, form one polycistronic cluster, mutations in NSHL patients were also analyzed in these two genes; however, no additional mutations were found in MIR182 or MIR183 [86,93].

The role of the MIR96 loss-of-function mutations in NSHL is consistent with the progressive hearing loss observed in the diminuendo mice with the induced miR-96-5p seed mutation, which resulted in the upregulation of several of the wild-type targets in the mutated mice. However, a parallel gain-of-function effect is also possible, as several genes in the mutated mice were downregulated in comparison to those in the wild-type animals [94]. Moreover, in heterozygous MIR96/MIR183 knockout mice, no serious alterations in hearing were observed (homozygotes were deaf), while in heterozygous diminuendo mice (with the seed mutation), hearing loss was observed. These findings further suggest a gain-of-function effect of this seed mutation [95].

5.2. MIR184 and eye diseases

Another miRNA gene mutation has been identified in MIR184 at the 4th nucleotide of the miR-184-3p seed sequence (n.57C>T) in two eye diseases. This mutation has been identified in a large Irish family affected by an autosomal-dominant form of severe keratoconus with early-onset cataract [96] and in a family with endothelial dystrophy, iris hypoplasia, congenital cataract, and stromal thinning (EDICT) syndrome, in which it clearly segregated with the disease phenotype [97]. Keratoconus is a heterogeneous disease characterized by progressive thinning and steeping of the cornea, with a prevalence widely ranging from 0.2 to 5000 per 100 000 people, depending on the diagnostic criteria or the ethnicity of the tested group [98]. Consistent with the pathogenesis of the diseases, miR-184-3p is the most abundant miRNA in the cornea, and it is expressed predominantly in the basal and suprabasal cells of the corneal epithelium and lens [99,100]. It plays a role in the neovascularization of the cornea [101] and the proliferation and differentiation of epidermal cells [102]. Previous studies have shown that the crucial function of miR-184-3p is competition for the binding sites with miR-205-5p and disabling the miR-205-5p to downregulate two of its targets, INPPL1 and ITGB4, playing a role in the pathogenesis of eye diseases, i.e., in the proper functioning of corneal basal epithelial hemidesmosomes and in keratocytes apoptosis, respectively [96,103]. This mutation stabilizes the structure of the miRNA precursor by reducing the internal loop size in miRNA duplex due to A:U base pairing instead of A:C mismatch. Functional in vitro experiments showed that the mutant miR-184-3p fails to block the miR-205-5p binding sites in its targets, thus enabling the downregulation of INPPL1 and ITGB4 by miR-205-5p [96]. Regardless of affecting the competition with miR-205-5p, the n.57C>T mutation may also affect the recognition/downregulation of the genuine miR-184-3p targets. For example, it was demonstrated in mouse and human keratocytes that the mutant miR-184-3p failed to repress KISS1 and FIH1 and to induce Notch activation [102], demonstrating another loss-of-function effect of this mutation.

Another two mutations, n.3A>G and n.8C>A, in MIR184 were found in Australian and British families with keratoconus. Both mutations are located in the 5p flank region and result in a level of miR-184-3p – by approximately 40 % for n.3A>G and almost complete reduction for n.8C>A. Both n.3A>G and n.8C>A mutations lead to the destabilization of the miRNA precursor structure, respectively, by changing the A:U to the wobble base pair G:U, and by introducing a mismatch in the miRNA duplex that enlarges the internal loop near the DROSHA cleavage site, and this may lead to a complete block of miRNA processing [104].

5.3. MIR204 and retinal dystrophy

Another ocular disease caused by a mutation in a miRNA gene is inherited retinal dystrophy (IRD). Linkage analysis in a large multi-generation British family and the subsequent sequencing of
A

**MIR96**

| Impact of mutation | pre-miRNA | miR-5p | miR-3p | reference |
|--------------------|----------|--------|--------|-----------|
| no change          | no change| no change| no change| Mencía 2009, Solda 2012 |

**MIR142**

| Impact of mutation | pre-miRNA | miR-5p | miR-3p | reference |
|--------------------|----------|--------|--------|-----------|
| no change          | no change| no change| no change| Solda 2012 |

B

| Impact of mutation | pre-miRNA | miR-5p | miR-3p | reference |
|--------------------|----------|--------|--------|-----------|
| no tested          | no tested| no tested| no tested| Trissl 2018 |

| Impact of mutation | pre-miRNA | miR-5p | miR-3p | reference |
|--------------------|----------|--------|--------|-----------|
| no tested          | no tested| no tested| no tested| not tested |

| Impact of mutation | pre-miRNA | miR-5p | miR-3p | reference |
|--------------------|----------|--------|--------|-----------|
| no tested          | no tested| no tested| no tested| not tested |
affected individuals revealed a mutation in MIR204 in the 4th position of the miR-204-5p seed sequence (n.37C>T). The mutation perfectly segregated with the family with this disease in an autosomal dominant manner [105]. IRD is a large group of heterogeneous disorders that manifest as the dysfunction or degeneration of photoreceptors, which leads to visual impairment or blindness [106]. It was shown in mice that miR-204-5p is detected at a higher level in the retinal pigment epithelium (RPE) and the ciliary body and at a lower level in the inner nuclear layer of the retina and in the choroid plexus in the brain [107]. miR-204-5p plays a crucial role in the development and proper function of the retina [108] and potentially has a neuroprotective effect on photoreceptor cells [109].

This mutation has a destabilizing effect on the miR-204 precursor structure. It replaces the Watson-Crick pair C:G in the wobble pair U:G in the miRNA duplex and does not cause any other structural alterations. The in vitro experiments showed that this mutation does not affect the level of the precursor and miR-204-5p, which suggests that it does not affect miRNA biogenesis. However, in silico predictions revealed a much higher number of potential targets for the mutant than for the wild-type miR-204-5p. The RNA-seq analysis of cells transfected with plasmids coding for the wild-type or the mutated pre-miR-204 showed that the mutation both affects the downregulation of the genuine targets of the wild-type miR-204-5p and downregulates predicted targets of the mutant miR-204-5p. Subsequent injection of mutant miR-204-5p into medaka fish (Oryzias latipes) induced phenotypes consistent with the genetic disease causing photoreceptor alterations, including reduced numbers of both cones and rods as a result of increased apoptosis. These findings suggest the predominant gain-of-function effect of the mutation, although causative mutation-specific targets have not been indicated [105].

5.4. MIR140 and skeletal dysplasia

Skeletal dysplasia is a very heterogeneous group of diseases containing more than 400 different disorders [110], which affect mostly cartilage and bones [111]. The prevalence of these diseases at birth is approximately 1–1.6:5 000 [112,113]. Skeletal dysplasia is an inherited genetic disease caused by mutations in numerous genes [110], including a recently identified mutation in MIR140 [114]. miR-140-3p is the most abundant microRNA in cartilage and its level is approximately tenfold higher than miR-140-5p in mouse cartilage [115,116], but both strands are functional. miR-140-3p regulates cartilage development and homeostasis. In knockout mice, a mild skeletal phenotype of the disease is observed [117].

The extensive sequence analysis of two families with skeletal dysplasia revealed the same heterozygous mutation, n.24A>G, in MIR140. In both families, the mutation occurred de novo, and in one family, it segregated with the disease (in the other family, it was not informative as the affected proband did not have offspring) [114]. This mutation is located at the 1st position of the miR-140-5p seed sequence. Mice with the mutation introduced by CRISPR/Cas technology (homozygotes and heterozygotes) developed several skeletal abnormalities consistent with the skeletal dysplasia symptoms in the analyzed patients. The level of miR-140-5p slightly increased in mice with the mutation in comparison to that in the wild-type mice, and the level of miR-140-3p decreased. The RNA-seq analysis revealed that genuine targets of the wild-type miR-140-5p were not efficiently silenced by the mutated miR-140-5p, whereas many new, predicted mutation-specific targets were effectively downregulated. Targets for the mutant miR-140-5p include many genes important for skeletal development and homeostasis, including Loxl3, Btg1, and Trps, as well as genes involved in the hypoxia response (e.g., Hif1a) or extracellular matrix genes (e.g., Col10a1). These data suggest that the n.24A>G mutation in MIR140 is both a loss- and gain-of-function mutation [114].

5.5. MIR17HG and Feingold syndrome type 2

Feingold syndrome is an autosomal dominant disease, manifested by microcephaly, learning disabilities, short stature, and brachymesophalangy, usually associated with mutations in the MYCN locus. The whole-genome comparative genome hybridization (CGA) analysis of 10 index patients with skeletal abnormalities consistent with Feingold syndrome but without mutation in MYCN revealed two different microdeletions (2.98 Mb and 165 kb long) in the 13q31-3q2 region. Both deletions segregated with the disease in the patients’ families and the minimal overlapping region of the deletions encompassed MIR17HG [119]. The gene coding miR-17–92 cluster known as OncomiR-1 due to its involvement in carcinogenesis and its oncogenic role in many types of cancers. In normal cells, miR-17–92 cluster is involved in lung, heart, skeletal, and immune system development [119]. Later, similar microdeletions from 165 kb to 17 Mb in 13q31-q32 were found in several other ethnically diverse patients/families with similar conditions, consistently named Feingold syndrome type 2 (FGLS2) [120,121]. Detailed functional studies showed that the deletions lead to a haploinsufficiency of MIR17HG and that no other gene is involved in the disease [118,121].

On the other hand, microduplications in 13q31, overlapping MIR17HG were found in several patients with symptoms such as tall stature, macrocephaly, developmental delay, and skeletal abnormalities [122].

6. Cancer somatic mutations in miRNA genes

6.1. MIR15A and MIR16-1 and chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is one of the most common types of leukemia, with a median diagnosis age of 70 years [123] and an incidence of 4.7 per 100 000 people per year in the USA [124]. Somatic deletions overlapping the 13q14 region was found in more than 50 % of patients with CLL [125,126]. Further analysis showed that MIR15A and MIR16-1, encoded at that locus, are either deleted or otherwise downregulated in 68 % of CLL patients [127].
However, the levels of both miR-15a-5p and miR-16–1–5p are very high in normal CD5+ lymphocytes [128], indicating an important role of the miRNAs in the functioning of CD5+ B cells. It was later shown that independent of the deletion, the miRNAs can also be downregulated by other mechanisms, including silencing due to the activity of histone deacetylases [129]. The deletion of this region was also observed in other types of cancers, such as mantle cell lymphoma (MCL) [126], multiple myeloma, and prostate cancer [130].

Subsequent in vitro and in vivo functional studies confirmed the tumor suppressor character of these miRNAs, among others demonstrating their proapoptotic properties manifested by targeting and downregulating a key antiapoptotic gene, BCL2, which is frequently increased in many cancers, including CLL. Consequently, the somatic deletion of the MIR15A/MIR16-1 cluster is the main mechanism of BCL2 upregulation and thus decreased apoptosis in CLL [128]. Other oncogenes that are regulated by miR-15a-5p and miR-16–1–5p are MCL1, CCND1, and WNT3A [131]. Interestingly, the analysis of CLL patients (n = 75) for point mutations in the MIR15A/MIR16-1 region revealed a germline substitution in the 3p-flank region (7 nucleotides downstream of MIR16-1 (n.7G>A) in two unrelated CLL patients. In both patients, somatic deletion of the other MIR15A/MIR16-1 allele (loss-of-heterozygosity) and a substantial reduction in the miR-16–1–5p level were also observed. Subsequent functional analyses showed that the mutation results in an increased level of the primary precursor of miR-15a/miR-16-1, and decreased levels of pre-miR-15a and pre-miR-16-1 as well as both mature miRNAs [132]. These findings suggest that the mutation affects miRNA biogenesis at the DROSHA processing step [133].

The identification of MIR15A/MIR16-1 in a region frequently deleted in CLL prompted further analyses of somatic copy number alterations of miRNA genes in cancer. First, it was shown that miRNA genes are overrepresented in fragile sites and other regions frequently altered in cancer, which suggests that miRNA genes may be frequently affected by copy-number alterations in cancer [134]. Subsequently, it was demonstrated that many miRNA genes undergo very high and frequent somatic copy number alterations, either deletions or amplifications, in different cancer types [135–137], suggesting a more global role of somatic copy number variation in the regulation of miRNAs in cancer.

6.2. MIR142 and blood cancers

The recent pan-cancer analysis, based on an analysis of data of greater than 10 000 cancer samples generated within The Cancer Genome Atlas (TCGA) [138] and Pan-cancer Analysis of Whole Genome (PCAWG) [139] projects, revealed that the most frequently mutated miRNA gene in all cancer types is MIR142 [with the highest frequency in diffuse large B-cell lymphoma (DLBCL)] [140]. Altogether, over 50 somatic mutations in MIR142 have been identified in different studies thus far [summarized in [140,141]]. Although the mutations are dispersed over the entire pre-miR-142 sequence (including the directly adjacent ~ 20-nucleotide-long flanking sequences), the mutations are enriched in the miR-142–3p seed sequence. Furthermore, the mutations in the 4th and 7th nucleotides (n.56G>N and n.59T>C; N – different substitutions) of the miR-142–3p seed sequence are the most frequent mutations. The distribution of the somatic mutations over the entire pre-miR-142 sequence and heterogeneity of the seed mutations (different positions of the 3p seed are mutated with different substitutions) strongly suggest the loss-of-function character of the mutations and tumor suppressor character of MIR142. Consistent with the function of miR-142–3p (and to a lesser extent miR-142-5p) in hematopoiesis, immune homeostasis, and the immune response that is exhibited mostly in the blood [142] and the high level of these miRNAs in peripheral blood cells [143], somatic mutations in MIR142 occur predominantly (almost exclusively) in hematologic malignancies, including acute myeloid leukemia (AML; 0.4–3.5 % of samples) [140,144,145], chronic lymphocytic leukemia (CLL; 0.5–1.1 %) [141,146], follicular lymphoma (FL; 9.5–25 %) [147–149], and DLBCL (12–27 %) [140,147,148,150]. However, single somatic mutations have also been detected in solid tumors, such as breast cancer, endometrial cancer, bladder cancer and glioblastoma [140].

It was demonstrated in different functional analyses that both miR-142-5p and 3p regulate many important hematologic targets (summarized in [141]), including Rac Family Small GTPase 1 (RAC1), TNFRSF13C encoding B-cell activating factor receptor (BAFF-R), interleukin 6 (IL6), and CD74 encoding programmed death-ligand 1 (PD-L1), an important immune checkpoint molecule. In knockout mice, aberrant development of marginal zone B cells, peripheral B1 B cells and T cells, dendritic cells, immunodeficiency, and impaired erythropoiesis have been found [151–153].

Despite the detection of mutations in different parts of MIR142, functional tests have been performed only for a few mutations located in the 3p seed sequence. As expected, the different seed mutations had slightly different effects on miRNA biogenesis and levels [150,152,154] and as shown in the structural modeling performed by us, the different seed mutations have a different effect on the structure of miRNA precursor (Table 1, Fig. 2B). For example, in vitro overexpression experiments and analysis of patient samples showed that mutations n.55A>G and n.58G>C, located in the 3rd and 6th positions of the miR-142–3p seed, do not affect the level of pre-miR-142, have little or no effect on the level of miR-142–3p, and substantially reduce the level of miR-142-5p [152]. The decrease in the miR-142-5p level most likely results from the destabilization of the miRNA duplex at the 3p side of miR-142-5p and preferential loading of miR-142-3p into the RISC. Regardless of the effect of the mutations on the miRNA level, both n.55A>G and n.58G>C, as well as n.59T>C and n.59T>A located at the 7th nucleotide of the seed sequence, disabled the recognition and downregulation of the well-recognized targets of miR-142–3p, RAC1, TGFBR1, ADCY9, and ASHL1 [150,152], confirming the loss-of-function character of the mutations. Subsequently, it was demonstrated in mouse models that inefficient downregulation of ASHL1 by the mutant miRNA results in increased levels of HOXA9 and A10 (positively regulated by ASHL1) and aberration in hematopoietic differentiation. This enhances myeloid differentiation and suppresses lymphoid lineages, ultimately leading to leukemic transformation and AML [152]. Independently, it was noticed, in a few AML patients, that MIR142 mutations co-occur with the hotspot mutations in IDH2 (R140), and it was demonstrated that the effects of MIR142 and IDH2 mutations synergize, leading to AML transformation [154].

It was also demonstrated that the novel miR-142–3p, resulting from the n.59T>C mutation may recognize and downregulate new mutation-specific targets, i.e., ZEB1 and ZEB2, playing a role in tumorigenesis. Thus, some of the mutations may also act as gain-of-function alterations [150].

6.3. Global analysis of cancer somatic mutations in miRNA genes

Thanks to the development of new sequencing technologies enabling whole-genome sequencing studies of large sets of cancer samples [155,156], many more cancer-driving mutations in non-coding regions, including miRNA genes, may emerge in the near future. The abovementioned pan-cancer analysis cataloged and characterized 7110 somatic mutations in 1179 miRNA genes in TCGA samples and 1523 somatic mutations in 856 miRNA genes in PCAWG samples [140]. The mutations were almost equally distributed in all parts of the miRNA precursors, including the miRNA
duplex (both within and outside of seed sequences), Dicer1/ Drosha cleavage sites, apical loop, and 25 nucleotides long upstream and downstream flanking sequences. It was demonstrated, with the use of different computational analyses, that many of the mutations may affect miRNA precursor structure and stability or distract existing protein binding motifs and that the seed mutations severely affect the predicted miRNA targets [17,140]. Although most of these mutations are most likely randomly occurring neutral variants, some of the miRNA genes were shown to be significantly overmutated across cancers or specific cancer types, and some were identified as potential cancer driver genes. However, it must be noted that future studies are needed to prove the functional potential of the identified mutations. Among these interesting examples (except the abovementioned MIR142) are (i) MIR205 predominantly mutated in melanoma, with a total of 17 mutations in pan-cancer, including hotspot mutation (n.35C>T) at the 1st nucleotide of miR-205-5p seed sequence, (ii) MIRLET7D with hotspot indels mutations in the upstream flanking sequence, (iii) MIR487B with 16 mutations and the hotspot mutation (n.54C>T) at the 3rd nucleotide of the miR-487b-3p seed sequence occurring in melanoma, and (iv) MIR122 with two mutations in the upstream 5p flanking sequence that were identified in liver cancer. All miRNAs encoded by these genes are well recognized as playing important roles in cancer. MIR122 has also been detected as an overmutated potential cancer driver in liver carcinoma in others genome wide studies utilizing different cancer driver identification strategies [156,157]. It is worth mentioning that MIR-122-5p is a highly liver-specific miRNA that plays many important regulatory roles in liver cells and is involved in the development of hepatocellular carcinoma [158].

7. Summary and outlook

Genetic variants located in miRNA genes lead to a wide range of changes in miRNA precursor structure and function by affecting the processing of the precursors (miRNA biogenesis) and the level of mature miRNAs, as well as by altering target recognition. To date, numerous SNPs associated with different diseases and disease-related conditions have been reported [21,37,38]. Moreover, mutations in miRNA genes, i.e., MIR96, MIR184, MIR204, and MIR140 and microdeletions of MIR17HG that have been implicated in hereditary disorders, and cancer germline and somatic mutations in MIR15A/MIR16-1 and MIR142 have been convincingly documented, strongly suggesting their disease- or cancer-driving potential. The examples cited in this article show that mutations in miRNA genes can affect miRNA genes in different ways, and while most mutations have a loss-of-function effect, some may also act as gain-of-function mutations.

We believe that the presented examples may help to interpret the consequences of these mutations and to better predict the effects of genetic variants that are identified in miRNA genes. However, we also have to admit that the available data are still very limited. Additionally, the ability to predict the effect of mutations in miRNA genes is far from that in protein-coding sequences, where the genetic code greatly helps to distinguish deleterious/-functional mutations from those of neutral or of uncertain character. The most important limitations of analyses of mutations in miRNA genes are briefly discussed below.

First, only a small fraction of variants identified in miRNA genes are functionally tested or otherwise characterized. For example, hundreds of SNPs [33] or thousands of cancer somatic mutations [17,140] have been identified in miRNA genes; however, only a few of them have been tested in any way. Second, functional testing is strongly biased toward analyses of mutations in seed sequences, which are considered to be the most crucial part of miRNA genes. Seed mutations have the most direct impact on target recognition/silencing, which is easier to demonstrate than the effects of mutations in other parts of miRNA genes. This ascertainment bias is also reflected in the examples presented in our study. However, seed mutations account for a small fraction of genetic variants in miRNA genes, i.e., ~4% of SNPs [33] and ~10% of cancer somatic mutations [17,140] identified in miRNA genes, and the higher functionality of seed sequences is not reflected in a higher conservation or substantially lower SNP density in seed sequences [33] compared to other parts of the miRNA hairpin. Third, most functional testing of miRNA gene mutations is performed with the use of simple in vitro tests based on very strong overexpression of artificial truncated sequences of miRNA genes and miRNA targets. Such analyses, although useful in primary screening, often may generate false-positive results. The effects of miRNA gene mutations are rarely tested in a natural sequence context and/or in patient samples. At least a partial solution for this limitation may be the generation of specific mutations with the use of CRISPR/Cas-based technologies. Fourth, there is still a lack of systematic functional analyses of sequence variants in miRNA genes. The only such study that analyze the effect of ~20 SNPs was performed over 10 years ago in pre-next generation sequencing era [36]. Fifth, most of the association studies of SNPs in miRNA genes are strongly underpowered (performed in small panels of samples) and rarely replicated in independent studies. They rarely meet the high standards of genetic association studies. Last, there are many hereditary and sporadic genetic diseases in which the exact etiology and causative variants are not known [159]; however, mutations in the non-protein-coding regions, including miRNA genes, are still considered a last resort.

Despite all limitations listed above, we believe that the recent extension of genetic analyses toward the whole genome, instead of concentrating on protein-coding sequences, will allow identification and a better understanding of genetic variants in miRNA genes as well as in other non-protein-coding parts of the genome. Of course, such a shift, except for the increased sequencing power, will require systematic functional and genetic analyses of a much wider number of mutations and the development of new molecular, computational and statistical methods that allow much more robust and high-throughput characterization of variants in miRNA genes, convincingly distinguishing functional deleterious mutations from neutral variants.

CRediT authorship contribution statement

Magdalena Machowska: Conceptualization, Visualization, Resources, Writing - original draft, Writing - review & editing. Paulina Galka-Marciniak: Conceptualization, Funding acquisition, Writing - review & editing. Piotr Kozłowski: Conceptualization, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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