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MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine

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Abstract

Referred to as the micromanagers of gene expression, microRNAs (miRNAs) are evolutionarily conserved small noncoding RNAs. Polymorphisms in the miRNA pathway (miR-polymorphisms) are emerging as powerful tools to study the biology of a disease and have the potential to be used in disease prognosis and diagnosis. Detection of miR-polymorphisms holds promise in the field of miRNA pharmacogenomics, molecular epidemiology and for individualized medicine. MiRNA pharmacogenomics can be defined as the study of miRNAs and polymorphisms affecting miRNA function in order to predict drug behavior and to improve drug efficacy. Advancements in the miRNA field indicate the clear involvement of miRNAs and genetic variations within the miRNA pathway in the progression and prognosis of diseases such as cancer, neurological disorders, muscular hypertrophy, gastric mucosal atrophy, cardiovascular disease and Type II diabetes. Various algorithms are available to predict miRNA-target mRNA sites; however, it is advisable to use multiple algorithms to confirm the predictions. Polymorphisms that may potentially affect miRNA-mediated regulation of the cell can be present not only in the 3'-UTR of a miRNA target gene, but also in the genes involved in miRNA biogenesis and in pri-, pre- and mature-miRNA sequences. A polymorphism in processed miRNAs may affect expression of several genes and have serious consequences, whereas a polymorphism in miRNA target site, in the 3'-UTR of the target mRNA, may be more target and/or pathway specific. In this review, we for the first time suggest a classification of miRNA polymorphisms/mutations. We also describe the importance and implications of miR-polymorphisms in gene regulation, disease progression, pharmacogenomics and molecular epidemiology.

Keywords

classification; diagnosis; disease; drug resistance; epidemiology; epigenetics; microRNA; miRSNP; mutations; pharmacogenomics; polymorphism; prognosis; SNP

MicroRNAs (miRNAs) are small, single-stranded, 21–23 nucleotide-long, independent functional units of noncoding RNA [1–3]. Often referred to as the ‘micromanagers of gene expression,’ miRNAs are evolutionarily well-conserved and, by binding to the target transcript in the 3'-UTR, can inhibit the translation of proteins and destabilize their target mRNAs [4–7]. Predicted to regulate almost a third of the human genome, miRNAs are essential for cellular and organism development [3,8]. The discovery of miRNAs, encoded in what was previously considered ‘junk DNA’, as master regulators of gene expression has revealed that the term

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'junk DNA' is a misnomer [9]. Over the past 8 years, the ongoing miRNA revolution has resulted in more than 4300 publications documented in PubMed alone [10]. To date, 678 human miRNAs have been characterized; however, computational predictions suggest that the total number of different miRNA sequences in humans may exceed 1000 [11–12], or even approach tens of thousands [13].

MiRNAs regulate specific genes broadly involved in multiple pathways such as cell death, cell proliferation, stress resistance and fat metabolism [14–16]. Biological functions of a few miRNAs have just begun to be more clearly understood. Unlike plants, in animals miRNAs were believed to act by causing translational repression rather than mRNA degradation. However, accumulating evidence now suggests that miRNAs not only inhibit translation of, but also destabilize its target mRNA [4,6,7]. Microarray analysis of human cells after transfections of miR-1 and miR-124 has revealed that approximately 100 target mRNAs are downregulated [17]. Recently it was demonstrated that a loss-of-miR24-function polymorphism in the dihydrofolate reductase (*DHFR*) 3'-UTR results in high steady-state levels of DHFR protein and mRNA levels, and a twofold increase in the half-life of the target mRNA [4], suggesting that in a mammalian cell, target mRNA destabilization may be a principle mechanism of miRNA action [4]. More recently, by using endogenous miRNA knockdown and miRNA transfections, it was demonstrated that approximately a third of the miRNA targets that are translationally repressed in a cell display mRNA destabilization [6,7]. Hence miRNAs fine-tune protein output in the cell by translationally repressing and destabilizing the target mRNA [4,6,7].

MiRNA genes are transcribed by RNA polymerase II, resulting in a hairpin-shaped pri-miRNA that is approximately 500–3000 bases long. The pri-miRNA is further processed by Drosha/Pasha to form a 60–70 nucleotide long pre-miRNA (Figure 1 & 2), which is transported from the nucleus to the cytoplasm through nuclear pore complexes with the help of Exportin-5/Ran-GTP. The pre-miRNA is further cleaved in the cytoplasm by an RNase III endonuclease, Dicer, to release two complementary short RNA molecules. The argonaut protein complex selectively binds to the guide strand and facilitates the formation of a miRNA–RNA-induced silencing complex (RISC) assembly [18]. Upon miRNA binding, the RISC complex is activated, and by a mechanism that is still unclear, locates its binding site in the 3'-UTR of the target mRNA and contributes to regulation of the gene's expression (Figure 1) [9,19–22].

More and more evidence suggests that a gain or loss of miRNA function is associated with disease progression and prognosis [4,23–28]. Several studies have now established that miRNAs are differentially expressed in human cancers as compared with the normal tissue [23,29–33]. Examples are: downregulation of two miRNAs, miR-143 and miR-145, in colorectal cancer [24]; increased expression of the miR-155 precursor in pediatric Burkitt lymphoma [25]; and down-regulation of Let-7 in lung cancers, associated with poor prognosis [26]. MiRNA-microarrays hold promise for cancer prognosis and diagnosis [27]. Some miRNAs have the potential to act as an oncogene or a tumor suppressor by affecting the expression of a tumor suppressor or an oncogene, respectively [34–36]. MiRNAs also play a role in cancer progression by regulating cell proliferation genes such as *c-MYC* and *E2F1* [28–31,37].

Polymorphisms in the miRNA regulatory pathway (miR-polymorphisms or SNPs that interfere with mRNA function [miRSNPs]) are a novel class of functional polymorphisms present in the human genome. MiR-polymorphisms reside at or near a miRNA binding site of a functional gene, influencing its expression by interfering with miRNA function [4,5,9,10]. In a relatively short time (less than 2 years) several groups worldwide have acknowledged the role of miR-polymorphisms, and suggested a strong association of miR-polymorphisms with disease progression and drug response.

In this review we first introduce miR-polymorphisms and discuss how a miR-polymorphism can affect expression of its target gene. Based on their functions and locations we next classify these miR-polymorphisms in different categories and further describe each category in detail. We also discuss the role of these polymorphisms in disease progression, diagnosis and prognosis. We highlight the promise of the miRNA pharmacogenomics field, and then conclude with the implications of miRNA polymorphisms to pharmacogenomics and epidemiology.

MiR-polymorphisms interfere with mRNA function

Generally, miRNAs regulate gene expression of a target gene by binding to its 3'-UTR. MiRNAs can potentially regulate expression of multiple genes and pathways; for example, it has recently been shown that the miR-15a/16-1 cluster can directly or indirectly regulate the expression of approximately 14% of known human genes [38]. A single miR-polymorphism can potentially affect the expression of multiple genes involved in pathways regulating drug absorption, metabolism, disposition, stem cell function and the cell cycle (Figure 3), and may affect the overall clinical efficacy of a drug or resistance to that drug (Figure 4). It has been demonstrated that a cell with a variant miRNA may be naturally selected [39]. Analysis of the publicly available SNP database revealed the presence of a relatively high level of variations in the 3'-UTRs of miRNA target genes [40]. However, relatively low levels of variation were observed in the miRNA seed region of a functional miRNA. Approximately 250 SNPs were found to potentially create target sites for miRNAs [40]. Functional polymorphisms in the 3'-UTRs of several genes have been reported to be associated with diseases by affecting gene expression. Some of these polymorphisms may interfere with the function of miRNA and are potential miR-polymorphisms able to affect the expression of miRNA targets [4,5,41,42].

Classifying miR-polymorphisms & mutations

A miRNA mutation can be defined as a mutation that interferes with miRNA function (hereafter miR-mutations). In a population, miR-polymorphisms and miR-mutations can be present either in heterozygous or homozygous form. In the human genome these polymorphisms can exist in the form of insertions, deletions, amplifications or chromosomal translocations, resulting in loss or gain of miRNA site/function [4] (Figure 3). A somatic cell in the human body can be profoundly influenced by a miR-mutation. A somatic miR-mutation can potentially alter cell morphology, induce cell death or contribute to carcinogenesis. A miR-mutation in a germline cell can be transmitted to the next generation resulting in an altered phenotype.

MiR-polymorphisms/miR-mutations can cause a gain or loss of miRNA function. Functional miR-polymorphisms or mutations can create or destroy a miRNA binding site within a target mRNA and affect gene expression by interfering with the function of a miRNA [4,5,9,10]. An example of a miRNA gain-of-function polymorphism is a G > A mutation in the *GDF8* allele of the myostatin gene in Texel sheep. The mutation creates a potential illegitimate miRNA target site for miR-1 and miR-206 and is associated with sheep muscular hypertrophy [43]. An example of a loss of miRNA function polymorphism is a C>T SNP present in the 3'-UTR of *DHFR*, preventing miR-24 binding. The increase or decrease in miRNA binding caused by a polymorphism may lead to a corresponding decrease or increase in protein translation [4,44]. For example, SNP-associated deregulation of the expression of an oncogene or tumor suppressor might contribute to tumorigenesis [35,36,45]. Based on the current knowledge of the field we suggest a classification for miRNAs (Table 1 and discussed below). MiRNA polymorphisms/mutations can be classified in the following three major categories:

- Polymorphisms or mutations affecting miRNA biogenesis
- MiR-polymorphism/mutations in miRNA target sites

- MiR-polymorphisms/mutations altering epigenetic regulation of miRNA genes.

Polymorphisms or mutations affecting miRNA biogenesis (see Table 1: A)

Several proteins and protein complexes are involved in various steps of miRNA biogenesis, such as miRNA transcription, processing, export and targeting. These proteins include RNA polymerase II complex, Drosha/Pasha, Exportin-5/Ran-GTP, nuclear pore complexes, Dicer and the Argonaute protein complex/RISC complex. Polymorphisms present not only in miRNA precursors but also in the proteins involved in its biogenesis may potentially affect miRNA-mediated regulation of the cell (Figure 1).

MiR-polymorphisms/mutations affecting microRNA biogenesis can be further sub-classified in following three categories:

- In pri- and pre-miRNA transcripts
- In mature miRNA sequences
- Affecting expression of the proteins involved in various steps of miRNA biogenesis

Polymorphisms/mutations pri-miRNA & pre-miRNA transcripts (see Table 1: A1)

Polymorphisms present in pri-, pre- and mature-miRNA can potentially influence expression of hundreds of genes and pathways, broadly affecting miRNA function. Sequence variations in miRNA genes, including pri-miRNAs, pre-miRNAs and mature miRNAs, could potentially influence the processing and/or target selection of miRNAs [46]. A bioinformatics approach was used to study 79 polymorphisms in the 3'-UTRs of 129 cancer associated genes, of which seven SNPs were found to be located in pre-miRNA hairpins and one in the miR-608 mature sequence [47]. In a screen of 227 known human miRNAs, a total 323 SNPs were identified, of which 12 were found to be located within the miRNA precursor [46]. A C>T germline alteration in the primary transcript of miR-15a/miR-16 was found in some patients with familial chronic lymphocytic leukemia (CLL) [31,48]. The polymorphism was found to be associated with reduced expression of miR-15 and miR-16. Approximately 70% of CLL cases express low levels of these two miRNAs, suggesting an association of this genetic polymorphism to leukemogenesis [31,48–50].

Recently a pre-miRNA SNP (rs11614913) in miR-196a2 was found to be associated with survival in individuals with non-small-cell lung cancer (NSCLC). A significant decrease in survival was observed in individuals homozygous for the SNP (CC), suggesting that the SNP could be a prognostic marker for NSCLC. This SNP was also shown to affect the binding of miR-196a to its target mRNA and resulted in a significant increase in mature miR-196a levels with no changes in the precursor miRNA, suggesting that the mature miRNA is directly processed from the pre-miRNA [51]. A more recent follow-up case-control study in Chinese women with breast cancer, identified the rs11614913 (T>C) polymorphism in miR-196a2 and an A>G SNP (rs3746444) in miR-499, associated with a significant increased risk of breast cancer susceptibility [52]. A common G>C polymorphism (rs2910164) in pre-miR-146a affects miRNA expression and contributes to the genetic predisposition to papillary thyroid carcinoma (PTC). Approximately 4.7% of PTC tumors have undergone somatic mutations of the SNP sequence, suggesting that the SNP plays a role in tumorigenesis through somatic mutation [53].

Polymorphisms in mature miRNA sequences (see Table 1: A2)

MiRNA binds to the target mRNA with Watson–Crick complementarity. Primarily a miRNA consists of two regions (see Figure 2): The 5'-region of a miRNA, from positions 2–7, called as the 'seed' region, which is thought to confer much of the target recognition specificity. The

other region of the miRNA, apart from the seed region, is able to tolerate mismatches to a certain extent; therefore, we coined the term 3'-mismatch tolerant region (3'-MTR) to describe this region. A miRSNP in miR-608 mature sequence has been identified *in silico* [47]. It was demonstrated in plants, (*Arabidopsis* and related *Brassicaceae*), that mutations in the miRNA itself resulted in loss of miR-319a function, which was further compensated by other members of the miR-391 family [54].

MiRNA polymorphisms/mutations in mature miRNA sequences can be further subclassified in following two categories:

- i. In miRNA 5'-seed region
- ii. In miRNA 3'-mismatch tolerant region (3'-MTR).

Polymorphisms in a mature miRNA seed region (see Table 1: A2i)—It has been suggested that the 5'-seed region is important for miRNA binding; however, this is not a reliable predictor of the actual miRNA target [55]. Since the miRNA seed sequences are short and highly conserved, the probability of a 'miRNA-seed polymorphism/mutation' are expected to be lower than a 'miRNA target site polymorphism/mutation'. Indeed, one study indicated that the likelihood of a SNP occurring in a miRNA seed region is less than 1% [40]. A recent study identified a polymorphism present in the seed region of miR-125a that significantly inhibited the processing of pri-miRNA to pre-miRNA, resulting in reduced miRNA-mediated translational repression [56]. A recent study suggested that experimental mutations in the 5'-seed of miR-206 (elevated in estrogen receptor (ER)- α -negative breast cancer) disrupted hybridization two miR-206 sites in hER- α -1 and hER- α -2 [57].

Thus, a SNP can either abolish or weaken a miRNA target, or create a perfect sequence match to the seed of a miRNA that otherwise was not associated with the given mRNA [44,58,59]. Although miRNA seed region polymorphisms can theoretically affect the expression of hundreds of genes, this prediction will require experimental validation. Moreover, the significance of miRNA seed region polymorphisms from the standpoint of population genetics has yet to be determined in large sets of cancer patients.

Polymorphisms in mature miRNA 3'-MTR (see Table 1: A2ii)—Unlike the mRNA seed region, which is very sensitive to mismatches, we predict that the 3'-MTR may tolerate mismatch SNPs to a certain extent, however, multiple SNPs, insertions, deletions or translocations in this region can potentially affect the miRNA mediated regulation of the target gene. However, this possibility needs to be further investigated.

Polymorphisms affecting the expression of the proteins involved in various steps of miRNA biogenesis (see Table 1: A3)

We propose that polymorphisms that affect expression of proteins involved in miRNA action and biogenesis, such as Drosha, Dicer, exportin5-ranGTP and the proteins in the RISC complex, may affect miRNA-mediated regulation within the cell (Figure 3). Since these proteins affect global miRNA biogenesis, genetic knockout of some of these proteins are lethal in mice. Polymorphisms that affect expression of the proteins would likely deregulate miRNA biogenesis and synthesis. In Kaposi's sarcoma herpesvirus (KSHV)-infected body-cavity-based lymphoma (BCBL)-1 cells, a naturally occurring polymorphism in a miR-K5 viral miRNA precursor stem-loop results in reduced processing by Drosha and, therefore, lower levels of mature miRNA expression [60]. Since less or more miRNA expression may have serious consequences in a cell, polymorphisms affecting the proteins involved in various steps of miRNA biogenesis can affect overall miRNA transcription, processing, export and targeting and may have deleterious effects in a cell.

MiR-polymorphism in miRNA target sites (see Table 1: B)

In contrast to the miR-polymorphisms in miRNA biogenesis, a miR-polymorphism located at the 3'-UTR of a target (coding) gene are more abundant in the human genome and have a more defined and limited range of effects. MiR-polymorphisms in miRNA target sites will impact only its encoded target-mRNA and its downstream effectors, hence, are more specific. A recent genome-wide association (GWA) study suggests that a gene that has more than two miRNA target sites will have increased expression variability as compared with a gene that is not regulated by a miRNA. The variability is further induced by SNPs in the miRNA target sites [61]. Thus, considering the large number of less conserved 3'-UTR target sequences they will potentially harbor a higher frequency of target miR-polymorphisms, and are potentially more important from an epidemiological standpoint, reviewed in [59,62]. Detailed examples of target miR-polymorphisms are discussed in 'role of miRNA polymorphisms in disease progression, diagnosis and prognosis' section (*via infra*) [4,9,43,53,55,57,63–74].

MiRNA polymorphisms/mutations in miRNA-target-mRNA sites can be further subclassified in following three categories:

- At a miRNA binding site
- Near a miRNA binding site

At a miRNA binding site (see Table 1: B1)

The generic 3'-UTR of a gene consists of a miRNA binding site, divided into a miRNA seed region binding site and a nonseed region binding site we refer to as the 3'-MTR binding site (Figure 2). We propose that a miRNA target site polymorphism can be of two types: a polymorphism in the 5' end of the miRNA target site, where the seed region of miRNA binds (see Table 1: B1i), and a polymorphism in the 3'-MTR binding site (see Table 1: B1ii). Since the miRNA seed sequence plays an important role in target recognition and binding, we predict that a polymorphism in this region may have a higher probability of affecting a miRNA function, as compared with a polymorphism that is present in 3'-MTR binding region. However this concept needs to be tested experimentally.

Near a miRNA binding site (see Table 1: B2)

We also propose that polymorphisms outside the miRNA target site can be of two types: a polymorphism in the target mRNA outside the miRNA target site affecting accessibility of the miRNA (see Table 1: B2i). Unlike DNA–protein interactions, mRNA–protein interactions are based on the presence or absence of secondary structure motifs in mRNAs. Most of the miRNAs binding sites in the 3'-UTRs of a target mRNA lack a complex secondary structure, thereby facilitating access for a miRNA [75]. Mutations that can create or abolish a secondary structure near a miRNA binding site may potentially influence miRNA-mediated translational repression of a target gene by affecting the accessibility of a miRNA to its binding site [76].

A polymorphism near a miRNA target site could disrupt the association of miRNA with other regulatory elements present in the 3'-UTR of the target transcript (see Table 1: B2ii). The length of the 3'-UTR of a target miRNA provides significant potential for miRNA-mediated, transcript-specific gene regulation, where a target gene can be regulated by more than one miRNA. Other than a miRNA binding site, a 3'-UTR harbors binding sites for cytoplasmic polyadenylation element (CPE) binding proteins and the hexanucleotide AAUAAA signal for cleavage and polyadenylation [9]. MiRNAs are shown to promote polyadenylation by interacting with cytoplasmic polyadenylation elements and other proteins or protein complexes within the 3'-UTR [77,78]. MiR-polymorphisms may potentially affect these interactions [79].

It has been demonstrated that under certain cellular conditions a stable secondary structure could be unfolded to provide access to a miRNA target site [80]. This miRNA mediated regulation can be exploited by a cell during stress response or in tissue specificity [80,81]. There is evidence that two miRNAs may bind to a target mRNA in coordination. Binding of miRNA to its target site may induce remodeling of the secondary structures in the neighboring regions, facilitating binding of miRNAs [81]. Hence, polymorphisms near a miRNA target site can potentially influence the accessibility of a miRNA–RISC complex by affecting the RNA structural motifs necessary for RNA–protein interaction. Further analysis of the interactions between miRNA and other regulatory elements present in 3'-UTRs will shed more light on the function of miRNA polymorphisms and will eventually establish 3'-UTR as a hotspot for pathology [4,82].

MiR-polymorphisms/mutations altering epigenetic regulation of miRNA genes (see Table 1: C)

Various miRNA genes are affected by epigenetic silencing due to aberrant hypermethylation. Epigenetic silencing of a miRNA was found to be an early and frequent event in the development of breast cancer. Aberrant hypermethylation of miR-9-1, miR-124a3, miR-148, miR-152 and miR-663 was observed in 34–86% of cases of 71 primary human breast cancer specimens [83]. MiR-polymorphism-mediated epigenetic alteration of miRNA regulation is a new, unexplored area of research. We propose that miR-polymorphisms or miR-mutations that can alter epigenetic regulation of a miRNA (methylation or acetylation) can be a mechanism of disease progression. Gain or loss of epigenetic regulation of an oncogene or a tumor suppressor, respectively, due to a miR-polymorphism or mutation, may have devastating effects in a cell.

Role of miR-polymorphisms in disease progression, diagnosis & prognosis

Recent advances in human genome research have provided a wealth of knowledge and revolutionized the field of molecular epidemiology and pharmacogenomics, which in turn hold great promise for individualized medicine. Advancements in the miRNA field indicate a clear involvement of deregulated miRNA gene signatures in cancers [29–33], such as papillary thyroid carcinoma [29], chronic lymphocytic leukemia [30,33] and breast cancer [84]. Recent GWA studies suggest that variations present in regulatory sites are more likely to be associated with disease and not the variations within coding region [62,85,86], and support the notion that DNA sequence variations associated with multiple human diseases may also interfere with functions of miRNAs [87]. Recently the miR-181 family of miRNAs was found to be upregulated in erythroid differentiation, and associated with the downregulation of homeobox genes, providing insights into leukemogenesis of the cytogenetically normal acute myeloid leukemia (CN-AML) molecular high-risk group [88]. Following are some of the common disorders found to be associated with miR-polymorphisms.

Neurological disorders

MiRNA genes frequently reside in fragile genomic regions that are deregulated in cancer [55]. A polymorphism (var321-*SLITRK1*) in the 3'-UTR of the human Slit and Trk-like 1 (*SLITRK1*) genes was implicated in Tourette's syndrome (TS) and attention deficit–hyperactivity disorder (ADHD). In human brain, the *SLITRK1* gene is expressed at high levels and has a role in neurite growth and TS. The var321-*SLITRK1* polymorphism in the 3'-UTR of the *SLITRK1* gene was found to strengthen an existing miR-189 target site, resulting in a more stringent regulation of the gene, which may be associated with TS [89].

In hereditary spastic paraplegia patients (HSP), two independent *in silico* studies identified three different polymorphisms present in the 3'-UTR of receptor expression-enhancing protein

1 (*REEPI*) [63,64]. Since these three polymorphisms are present in the conserved nucleotides within the binding sites of two miRNAs, miR-140 (606+50G/A, 606+43G/T) and miR-691 (606+14C/T), it was inferred that these miRSNPs may disrupt weak G:U wobble base pairing and have the potential to affect miR-140- and miR-691-mediated regulation of *REEPI* [63, 64].

It is known that deletion of the 5-hydroxytryptamine (serotonin) receptor 1B (*HTR1B*) gene in mice results in aggressive behavior [65]. Recently, in a study utilizing 359 college students, it was demonstrated that an A>G polymorphism (rs13212041) in the *HTR1B* mRNA, affecting miR-96-mediated regulation of the gene, was found associated with aggressive behavior. Individuals homozygous for the ancestral A were reported to exhibit more conduct-disorder behaviors than individuals with the G element [65].

Several association studies in Parkinson's disease (PD) identified a polymorphism (rs12720208) in the 3'-UTR of fibroblast growth factor 20 (*FGF20*) as a risk factor for the disease. A recent study of a sample of 729 nuclear families with 1089 PD-affected and 1165 PD-unaffected individuals, suggested that the PD risk allele for rs12720208 disrupts a binding site for miR-433, increasing translation of *FGF20* *in vitro* and *in vivo*. Increased levels of *FGF20* are associated with PD, suggesting that individuals with the PD risk allele (rs12720208) may be predisposed to PD [66].

Muscular hypertrophy & cardiovascular diseases

As mentioned, in Texel sheep, it was demonstrated that a G>A mutation in the *GDF8* allele of the myostatin gene creates a potential illegitimate miRNA target sites for miR-1 and miR-206 that are highly expressed in skeletal muscle. The mutation causes translational inhibition of the myostatin gene and hence contributes to the muscular hypertrophy of Texel sheep [43].

An 1166A>C miRNA polymorphism (rs5186) in the angiotensin receptor 1 (*AGTR1*) gene was implicated in hypertension and cardiovascular disease [67,68]. The polymorphism was shown to abrogate the miR-155 mediated regulation of the *AGTR1* gene, resulting in downregulation of the *AGTR1* protein. Since *AGTR1* gene overexpression results in hypertension, this polymorphism is therefore implicated in hypertension and cardiovascular disease [67]. Hence the 1166A>C SNP may abrogate regulation by miR-155 and this may result in *AGTR1* overexpression and may explain the association of this polymorphism with hypertension [68].

Cancer

We have discussed (*vide supra*) the association of pre-miRNA polymorphism with cancers such CLL, C>T germline alteration in pre-miR-15a/miR-16 [31,48–50]; NSCLC, a SNP in pre-miR-196a2 [51]; PTC, a G>C SNP in pre-miR-146a [52]; and breast cancer, two SNPs T>C in pre-miR-196a2 and A>G in pre-miR-499 [53]. Moreover, miR-polymorphisms present in a miRNA target site more directly affect the function of a miRNA. Following are some examples of the miR-polymorphisms in the target mRNA that were found to be associated with cancer.

Colorectal cancer—A case-control study in the Czech population, which has the highest worldwide incidence of colorectal cancer, identified polymorphisms within miRNA-binding sites with a positive association with a risk of sporadic colorectal cancer [69]. In the 3'-UTR of cluster of the differentiation 86 (*CD86*) gene, a C>G polymorphism (rs17281995) predicted to affect miR-337, miR-582, miR-200a, miR-184 and miR-212 was significantly associated with colorectal cancer. The study also identified rs1051690 in insulin receptor (*INSR*) predicted to affect miR-618 and miR-612 [84].

Papillary thyroid carcinoma—A total of two polymorphisms were identified, in the miR-221/222 and miR-146a/146b miRNA binding sites in v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene homolog (*KIT*) gene, associated with deregulated expression of the KIT protein contributing to papillary thyroid carcinoma [29].

Breast cancer—A C>T (rs93410170) miRSNP in the 3'-UTR of ER- α , resulted in stringent miR-206 mediated regulation of ER- α . Since ER- α overexpression is associated with higher risk for breast cancer, it was suggested that the SNP may be associated with breast cancer [70]. An integrin- β 4 (*ITGB4*) SNP may influence breast tumor aggressiveness and survival, and it may have prognostic value in the clinic [85]. A chromosomal translocation that was found to be associated with human tumors was shown to disrupt the let-7 miRNA mediated regulation of an oncogene, high mobility group A2 (*HMGa2*). The disruption of a single miRNA-target interaction resulted in oncogenic transformation as assayed by anchorage-independent growth in soft agar [71]. Recently an epidemiologic study demonstrated the association of miRNA-related genetic variants may affect bladder cancer risk [72].

Gastric mucosal atrophy & irritable bowel syndrome

A polymorphism of miR-27a genome region was found to be associated with the development of gastric mucosal atrophy in Japanese male subjects [73]. Diarrhea predominant irritable bowel syndrome (IBS-D) is generally associated with dysfunctions in the serotonergic system. Recently a case-control study identified a variant c.*76G>A (rs62625044) in the 3'-UTR of serotonin receptor type 3 subunit gene (*HTR3A*), which showed a strong association with female IBS-D. Using a reporter assay, it was suggested that the c.*76G>A variant affected binding of miR-510 to the *HTR3E* 3'-UTR, resulting in high expression of the receptor subunit [74].

Type II diabetes

Recently, an ACAA-insertion/deletion polymorphism, associated with Type II diabetes, was found to be present in between the miR-657 and miR-453 binding sites within the 3'-UTR of human insulin-like growth factor 2 receptor (*IGF2R*). Loss of miR-657 mediated regulation, due to the polymorphism, results in deregulation of *IGF2R* gene expression and may provide the underlying mechanism as to how the ACAA polymorphism deregulates expression of the *IGF2R* gene [90].

MiR-polymorphisms alter drug response; miRNA pharmacogenomics

Recently, the role of miRNA in drug-resistance/sensitivity was realized [4,5,9,10]. It was functionally demonstrated that a polymorphism in a miRNA binding site could lead to drug-resistance/drug sensitivity. The term miRSNP/miR-polymorphism was coined and defined as a novel class of SNPs/polymorphisms that interfere with the function of miRNA [4,5,9,10].

A C>T SNP present in the 3'-UTR of *DHFR* was originally identified, in a case-control study of childhood leukemia patients, to occur with 14.2% allelic frequency in the Japanese population [91]. Later it was demonstrated that the SNP is present near a miR-24 miRNA-binding site in human *DHFR* [4]. The C>T SNP near the miRNA-binding site acts as a loss-of-function mutation and interferes with miR-24 function. The loss of miR-24-function results in high steady-state levels of *DHFR* mRNA and protein levels and results in drug resistance [4,5]. Of interest, loss of miR24 function due to the SNP resulted in a twofold increase in half-life of the target mRNA. This observation not only explained the corresponding increase in *DHFR* mRNA and protein levels but also suggested that the target mRNA destabilization could be a principle mechanism of action of a miRNA [4]. This finding may also be useful in predicting the clinical outcome of methotrexate treatment in the clinic.

A model describing the mechanism of action of a miR-polymorphism

By using drug response as a model, a mechanism of action of a miR-polymorphism is illustrated in Figure 4. MiR-polymorphisms, interfering with miRNA binding and cellular function, may result in increased translation of the ‘drug-target protein’ (a protein that is directly inhibited by binding of a drug) and may lead to drug resistance [4,5,9,10]. If the protein is a ‘drug-effector’ (a protein that enhances the effect of a drug), increased levels resulting from a miR-polymorphism will result in drug sensitivity. The model may also apply vice-versa – if a miR-polymorphism causes a gain of miRNA function, it could result in the downregulation of either a drug-target or a drug-effector protein, resulting in drug sensitivity or drug resistance, respectively (Figure 4). Various miR-polymorphisms located in many important genes that are drug targets may affect drug response in patients and may lead to drug resistance or drug sensitivity, and even unanticipated toxicity (Figure 4) [4,5,9,10].

MiRNA pharmacogenomics

Pharmacogenomics of miRNA is a novel and promising field of research that holds new possibilities for tailor-made medical therapy. MiRNA pharmacogenomics can be defined as the study of miRNAs and polymorphisms affecting miRNA function in order to predict drug behavior and to improve drug efficiency [9,10]. MiRNA pharmacogenomics has strong clinical implications for several reasons: miRNAs are attractive drug targets, are differentially expressed in malignant versus normal cells and regulate expression of several important proteins in the cell [49,81,84]. MiR-polymorphisms can interfere with miRNA function resulting in loss of the miRNA-mediated regulation of a drug-target gene conferring drug resistance [4]. Therefore, these miR-polymorphisms have potential as predictors of drug response in the clinic and will result in development of more accurate methods of determining appropriate drug dosages based on a patient’s genetic makeup, thus decreasing the likelihood of drug overdose [10].

Implications of miR-polymorphisms to pharmacogenomics & epidemiology

MiRNAs are associated with disease progression and can be used in the clinic to predict drug prognosis. MiR-polymorphisms have been shown to affect drug response and have the potential to confer drug resistance (Figure 4) [9]. A GWA study of human SNPs has revealed the association of several polymorphisms in the miRNA binding sites [40]. Some of these variations may have biological relevance and are worth further investigation in case-control studies examining their association with certain biological or pathological events. Some of the recent case-control studies have revealed the important role of miR-polymorphisms; these polymorphisms should be further functionally characterized.

Table 2 lists some of the functional algorithms that are available on the web to predict miRNA-targets and the evolutionary conservation of a miRNA and its binding site. Each of these algorithms follows distinct criteria to predict a miRNA-target sequence. A miRNA-target mRNA site predicted by one program many times is not the same as that predicted by another algorithm. Hence, it is advisable to use multiple algorithms to predict a miRNA binding sites on the target mRNA [39,92,93].

MiRNA-dependent target repression requires both sequence similarities and target accessibility. The best method to identify a miR-polymorphism is to combine computational predictions and functional studies. Evolutionary conservation of a miRNA binding site may be a key to help identify a valid miRNA binding site; however, the length of the 3'-UTR has increased with evolutionary age and the human 3'-UTRs, which are the longest, may hold some novel miRNA sites that would otherwise be ignored by the conservation algorithms. Also the length of 3'-UTRs provide significant potential for miRNA-mediated transcript-specific gene

regulation, where a target gene can be regulated by several miRNAs and by various other regulatory elements in coordination. Recently it was demonstrated that proliferating cells tends to express mRNAs with shortened 3'-UTRs and fewer miRNA target sites as compared with the nonproliferating cells [94]. Further analysis to how functional miR-polymorphisms affect the interactions between miRNA and other regulatory elements present 3'-UTRs will shed more light on the functional aspect of miR-polymorphisms and will establish the 3'-UTR as a hot spot for epidemiology and individualized medicine.

Future perspective

In the past 2 years alone, over 2000 miRNA sequences from 58 different species were submitted to the miRBase, the central online repository for miRNA, making a total of 5071 miRNA loci, expressing 5922 distinct mature miRNA sequences [12]. Many of these miRNAs may be clade- (a taxonomic group with a single common ancestor) or even organism-specific [17–19]. Since polymorphisms in a miRNA pathway can result in the loss or gain of a miRNA function and can affect expression of hundreds of genes, this new class of polymorphisms may provide clues to many unanswered fundamental questions associated with disease and evolution. There are fewer genes and proteins than anticipated in the genome of humans and other species; how then can we account for the various races or the many species that exist today? Since cells with mutant miRNA with altered functions are almost certainly subject to selective pressures, miR-polymorphisms may introduce a whole new perspective to the theory of evolution and may explain wide differences between races and also between species. In the future polymorphisms in miRNA target sites may become one of the largest forms of variation present among species.

We propose in Table 1 and Figure 3 that various types of miR-polymorphisms exist in the human genome. However, each type of miR-polymorphism and its effects needs to be experimentally verified. These polymorphisms may potentially influence miRNA biogenesis and target regulation and may have deleterious effect on the cell and the individual.

Other than polymorphisms, various miRNA genes affected by epigenetic silencing due to aberrant hypermethylation were found to be an early and frequent event in breast cancer development. In this review we also propose that miR-polymorphisms can alter epigenetic regulation of a miRNA and can be a mechanism of disease progression. MiR-polymorphism-mediated epigenetic alteration of miRNA regulation is a new, unexplored area of research.

Hence it seems that silencing miRNA-mediated regulation is a common mechanism involved in disease progression and may result in abnormal function of the cell. Cells with abnormal miRNA functions could be selected and amplified, contributing to diseases. Thus miR-polymorphisms are emerging as a powerful tool to study the biology of a disease, and have tremendous potential to be used in disease prognosis and diagnosis. Understanding the role and functions of miR-polymorphisms has a promising future in pharmacogenomics, molecular epidemiology and individualized medicine.

Executive summary

- Generally, miRNAs regulate gene-expression or a target gene by binding to its 3'-UTR. MiRNAs can potentially regulate expression of multiple genes and pathways.

MicroRNA-polymorphisms/-SNPs/-mutations can interfere with microRNA function

- A cell with a variant microRNA (miRNA) may be naturally selected. A sequence variation in the miRNA pathway can affect the expression of multiple genes and may affect the overall clinical efficacy of a drug.

Classifying miR-polymorphisms & mutations

- In a population, miR-polymorphisms can be present either in a heterozygous or homozygous configuration, in the form of insertions, deletions, amplifications or chromosomal translocations, resulting in loss or gain of a miRNA site/function.
- Polymorphisms affecting mi-RNA biogenesis such as polymorphisms in pri- and pre-miRNA transcripts; polymorphisms in mature miRNA, including miRNA 5'-seed region and miRNA 3'-mismatch tolerant region (3'-MTR); polymorphisms affecting expression of the proteins involved in various steps of miRNA biogenesis in such as transcription, processing, export and targeting.
- At or near a miRNA target site such as polymorphisms at miRNA binding region, where the seed region of miRNA binds and where 3'-MTR of a miRNA binds; polymorphisms near miRNA binding region, affecting the accessibility of a miRNA-RISC complex and affecting the coordination of miRNA with other regulatory elements present in 3'-UTR of the target transcript.
- Altering epigenetic regulation of miRNA genes: in this review we propose that polymorphisms affecting acetylation and methylation of genes will have a broad impact.

Role of miR-polymorphisms in disease progression, diagnosis & prognosis

- Neurological disorders: Tourette's syndrome and attention deficit-hyperactivity disorder patients: a polymorphism in *SLITRK1* gene strengthens an existing miR-189 target site; Henoch-Schonlein Purpura patients: three polymorphisms (i.e., 606+50G/A, 606+43G/T in miR-140 and 606+14C/T in miR-691) affect expression of *REEP1* gene; aggressive behavior: A>G polymorphism, affecting miR-96 mediated regulation of the *HTR1B* gene.
- Muscular hypertrophy: in Texel sheep, a G>A mutation in *GDF8* allele of the myostatin gene, creates a potential illegitimate miRNA target sites for miR-1 and miR-206 and downregulation of myostatin gene.
- Hypertension and cardiovascular disease: An 1166A>C polymorphism affects miR-155 mediated regulation of the *AGTR1* gene.
- Cancer: pre-miRNA polymorphism associated with cancers such as chronic lymphocytic leukemia, non-small-cell lung cancer, papillary thyroid carcinoma and breast cancer are reported. Colorectal cancer: Eight polymorphisms within miRNA-binding sites were found to have a positive association with a risk of sporadic colorectal cancer. Papillary thyroid carcinoma: *KIT* gene, two polymorphisms in the miR-221/222 and miR-146a/146b miRNA binding sites. Breast cancer – *ITGB4* SNP may influence breast tumor aggressiveness and survival, and it may have prognostic value in the clinic. A chromosomal translocation disrupts the let-7 miRNA mediated regulation of *HMGa2* oncogene.
- Gastric mucosal atrophy: a polymorphism of miR-27a genome region was found to be associated with the development of gastric mucosal atrophy in Japanese male subjects.
- Type II diabetes: ACAA-insertion/deletion polymorphism, associated with Type II diabetes, was found to be present in between the miR-657 and miR-453 binding sites within the 3'-UTR of *IGF2R*.

MiR-polymorphisms alter drug response

- The role of miRNA in drug-resistance/sensitivity was recently realized in a study that demonstrated that a miRSNP 829 C-T, present in the *DHFR* gene, near a miR-24 binding site, was associated with methotrexate drug resistance/sensitivity. The SNP is

present with a 14.2% allelic frequency in the Japanese population. The finding may be useful in predicting the clinical outcome of methotrexate treatment in the clinic.

MiRNA pharmacogenomics

- MiRNA pharmacogenomics can be defined as the study of miRNAs and polymorphisms affecting miRNA function in order to predict drug behavior and to improve drug efficiency. MiR-polymorphisms could be potential predictors of drug response in the clinic and may provide more accurate methods of determining appropriate drug dosages based on a patient's genetic makeup, thus decreasing the likelihood of drug overdose.

Implications of miR-polymorphisms to pharmacogenomics & epidemiology

- A combination of the computational predictions and the functional studies are needed to validate a functional miR-polymorphism. Thus miRNAs and miR-polymorphisms are powerful tools to study disease progression and can be used in the clinic to predict drug prognosis.

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Figure 1. MiRNA biogenesis and function

(A) A miRNA gene is transcribed by RNA polymerase II, resulting in a hairpin-shaped pri-miRNA that is approximately 500–3000 bases long. (B) The pri-miRNA is further processed by Drosha/Pasha to form a 60–70-nucleotide long pre-miRNA, (C) which is transported from the nucleus to the cytoplasm with the help of Exportin-5/Ran GTP through nuclear pore complexes. (D) The pre-miRNA is then identified and further cleaved in the cytoplasm by an RNase III endonuclease, Dicer (E), to release two complementary short RNA molecules (F). (G) The argonaut protein complex selectively binds to the guide strand and facilitates the formation of the miRNA–RISC assembly [18]. (H) Upon miRNA binding the RISC complex is activated and, by a mechanism that is still unclear, locates its binding site in the 3'-UTR of the target mRNA contributes to regulation of gene expression by translation inhibition and/or mRNA degradation [4,19–21]. Polymorphisms involved in any of these eight steps can potentially affect miRNA-mediated regulation of the cell.

MiRNA: MicroRNA; RISC: RNA-induced silencing complex.

Figure 2. MiRNA–mRNA hybrid regions

Using the human let-7 family of miRNAs (let-7a-g and i), we compare the pre- and the processed miRNA regions. (A) MiRNA primarily consists of two regions: the 5'-region of a miRNA, from positions 2–7, called the 'seed' region, which is thought to confer much of the target recognition specificity; and the miRNA region, other than seed region, which is able to tolerate mismatches to a certain extent. To refer to the 3'-region of a miRNA, other than the seed region, we coin the term 3'-MTR. MiRNA binds to the target mRNA with Watson–Crick complementarity. Unlike the miRNA 3'-MTR, the seed region is very sensitive to mismatch. Although very rare, a miRNA seed region polymorphism has a potential to affect the expression of hundreds of target genes. On the other hand, polymorphisms in the target mRNA, where the miRNA-seed region binds, can potentially affect the miRNA-mediated regulation of individual target genes. Although the 3'-MTR of a miRNA can tolerate SNPs to a certain extent, multiple SNPs, insertions, deletions or translocations in this region, can potentially affect the miRNA-mediated regulation of the target gene. (B) A few pre-miRNA precursors of the human let-7 miRNA family are shown (using MiRNAMP, see Table 2 for details). Although variations in the 3'-MTR exist within the let-7 miRNA family of miRNAs, all the members have a unique conserved seed sequence (GAGGUA).

MiRNA: MicroRNA; MTR: Mismatch tolerant region.

Figure 3. MiR-polymorphisms affecting miRNA function

We predict that polymorphisms present in the target mRNA, pri-miRNA, pre-miRNA, processed miRNA, Drosha, Dicer, exportin5-ranGTP and in the RISC complex may affect miRNA-mediated regulation in the cell. The miR-polymorphisms can be present in the form of insertions, deletions, amplifications, chromosomal translocations and so on, leading to loss or gain of a miRNA site/function [9]. The miR-polymorphisms could exist in either a heterozygous or homozygous state within a population. Since miRNAs are predicted to regulate genes involved in multiple pathways [14], miR-polymorphisms may affect the miRNA-mediated regulation of genes involved in cell death, cell proliferation, stress resistance and altered fat metabolism, and may potentially contribute to diseases and drug response [9]. MiRNA: MicroRNA; RISC: RNA-induced silencing complex.

Figure 4. A model whereby miR-polymorphisms influence drug response

The miRNA binds to the RISC and the resulting complex locates and binds to the 3'-UTR of a drug target gene (a gene product that is directly inhibited by binding of a drug), regulating its expression and resulting in less net drug-target protein in the cell. MiRNA polymorphisms can also interfere with miRNA binding and function, resulting in increased translation of the drug-target protein in cells that express the polymorphism, leading to drug resistance [4,5,9, 10]. If the miRNA target protein is a 'drug-effector protein' (a protein that enhances the effect of a drug), its increased level resulting from a miR-polymorphism will result in drug sensitivity. Vice versa will also be true: if a miR-polymorphism results in a gain of miRNA function, it

will cause downregulation of both drug-target and the drug-effector proteins resulting in drug sensitivity and drug resistance, respectively.

MiRNA: MicroRNA; RISC: RNA-induced silencing complex.

Table 1
Suggested classification of miR-polymorphisms and -mutations.

Types of miR-polymorphisms or -mutations	miR-polymorphisms	miRNA, target	Association	Ref.
(A) Affecting miRNA biogenesis (broad impact[*])				
1) In pri- and pre-miRNA transcripts	C>T	MiR-15/16	Leukemia	[31,48]
i) Pri-miRNA transcript	12 SNPs	Multiple		[46]
ii) Pre-miRNA hairpin	Seven SNPs	Multiple		[47]
		MiR-196a		[51]
		MiR-146a		[52]
		MiR-196a2		[53]
		Viral miR-K5	Affects Drosha processing	[60]
2) Polymorphisms in mature miRNA (rare, broad impact)	SNP	MiR-608		[47]
i) miRNA 5'-seed region	Mutation	Plant miR-319a		[54]
ii) miRNA 3'-MTR	Seed SNP	MiR-125a		[56]
3) Affecting expression of the proteins involved in various steps of miRNA biogenesis	ND	ND	ND	ND
i) Transcription				
ii) Processing				
iii) Export				
iii) Targeting				
(B) MiRNA target site polymorphisms (abundant, narrow impact[†])				
1) At a miRNA binding site	var321-SLITRK1	MiR-189, SLITRK1 miR-140, REEP1	Tourette's syndrome	[89]
i) Where the seed region of miRNA binds	606+50G/A, 606+43G>T	MiR-140, REEP1	Hereditary spastic paraplegia 31	[63]
ii) Where 3'-MTR of a miRNA binds	G>A (Sheep)	MiR-1, 206, myostatin	Hereditary spastic paraplegia 31	[63]
	829 C>T	MiR-24, DHFR	Sheep, muscular hypertrophy	[43]
2) Near a miRNA binding site	606+14C>T	MiR-691, REEP1	Drug-resistance	[4,5,9,10]
i) Affecting the accessibility of a miRNA-RISC complex.	1166A>C (rs5186)	MiR-155, AGTR1	Hereditary spastic paraplegia 31	[64]
ii) Affecting the coordination of miRNA with other regulatory elements present in 3'-UTR of the target transcript.	A>G (rs13212041)	MiR-96, HTR1B	Hypertension	[67,68]
	C>T(rs93410170)	MiR-206, ER- α	Aggressive behavior	[65]
	rs12720208	MiR-433, FGF20	Breast Cancer	[57]
	C>G (rs17281995)	MiR-337, 582, 200a, 184 and	Parkinson's disease	[66]

Types of miR-polymorphisms or -mutations

miR-polymorphisms	miRNA, target	Association	Ref.
C>G (rs17281995)	212, CD86	Colorectal cancer	[70]
rs1051690	MiR-618, 612, INSR	Colorectal cancer	[69]
Two SNPs KIT	miR-221/222,146a/b	Colorectal cancer	[69]
SNP rs743554	ITGB4	Papillary thyroid carcinoma	[29]
Translocation	Let-7, HMGA2	Breast cancer	[57]
Polymorphism	MiR-27a	Breast cancer	[71]
G>A (rs62625044)	MiR-510, HTR3A	Gastric mucosal atrophy	[73]
ACAA-ins/del	MiR-657, IGF2R	Irritable bowel syndrome	[74]
		Type II diabetes	[90]

(C) Altering epigenetic regulation of miRNA genes[§] (broad impact^{*})

i) Methylation	ND	ND	ND
ii) Acetylation			

* Broad impact: affecting expression of hundreds of genes.

‡ Narrow impact: affecting expression of only few genes, more gene/pathway specific effect.

§ In this review we propose that a miR-polymorphism or miR-mutation can potentially alter epigenetic regulation of a miRNA and can be a mechanism of disease progression.

3'-MTR: 3'-mismatch tolerant region of a miRNA; AGTR1: Angiotensin receptor 1; CD86: Cluster of differentiation 86; DHFR: Dihydrofolate reductase; ER- α : Estrogen receptor- α ; FGF20: Fibroblast growth factor 20; HMGA2: High Mobility Group A2; HTR1B: 5-hydroxytryptamine (serotonin) receptor 1B; HTR3A: Serotonin receptor type 3 subunit; IGF2R: Insulin-like growth factor 2 receptor; INSR: Insulin receptor; ITGB4: Integrin β 4; MiR: MicroRNA; MTR: Mismatch tolerant region; MTX: Methotrexate; ND: Not discovered yet (proposed); REEP1: Receptor expression-enhancing protein 1; SLITRK1: Human Slit and Trk-like 1.

Table 2

List of algorithms to predict miRNA target interaction.

Algorithm (URL)	Brief description of the algorithm
MiRBase (http://microrna.sanger.ac.uk/)	Includes a searchable miRNA sequence database, a miRBase registry with miRNA gene nomenclature, and a miRBase targets database with predicted miRNA targets in animals [12]
MiRanda (http://cbio.mskcc.org/mirnaviewer/)	Developed at the Computational Biology Center of Memorial Sloan-Kettering Cancer Center (NY, USA) miRanda is an algorithm for finding genomic targets for miRNA, provides evolutionary conservation of the target mRNA, sequence complementarity of the mature miRNA and the target site, the binding energy of the miRNA–target duplex [95]
TargetScan (http://www.targetscan.org/)	TargetScan is also a miRNA target prediction algorithm that predicts biological targets of miRNAs by searching for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA [96]
PicTar (http://pictar.bio.nyu.edu/)	PicTar computes a maximum likelihood score that indicates to what extent a given mRNA 3'-UTR is targeted by a fixed set of miRNA [95]
DIANA-MicroT (http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi)	Finds conserved miRNA target duplexes in human and mice with the least free-energy [97]
PolymiRTS database(http://compbio.utmem.edu/miRSNP/help.php#search)	PolymiRTS database is a collection of naturally occurring DNA variations in putative miRNA target sites and integrates sequence polymorphism, phenotype and expression microarray data [98]
MicroInspector (http://mirna.imbb.forth.gr/microinspector/)	MicroInspector generates a list of possible target sites, which are then sorted by free-energy values [99]
Patrocles(http://www.patrocles.org/Patrocles.htm)	Compiles SNPs in the 3'-UTR of human and mouse genes that either create or destroy miRNA target sites [43]
MiRNAMP (http://mirnamap.mbc.nctu.edu.tw/)	Provides miRNA expression profiles (tissue specificity and the differential expression in cancer/normal cells)

A miRNA-target mRNA site predicted by one program most of the time may not be the same as predicted by another algorithm. Hence, it is advisable to use multiple algorithms to predict a miRNA binding site on the target mRNA [34,70,71].