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# Impact of MicroRNAs in Cancer Research

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MicroRNAs (miRNAs) are a family of endogenous ~22nt non-coding RNAs that regulate gene expression with a strong sequence specificity. Since their discovery as regulators of developmental timing in *Caenorhabditis elegans* in 2001 (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001), miRNAs have been recognized to form one of the major regulatory gene families by targeting mRNAs for cleavage or translational repression within viruses, plants and animals.

MiRNA expression patterns were found to classify cancer types with a unexpected greater reliability than the respective profiles of protein-coding genes (Lu *et al.*, 2005; Rosenfeld *et al.*, 2008). This outstanding potential as diagnostic biomarkers is furthermore supported by the extraordinary stability of miRNAs in routinely collected formalin-fixed paraffin-embedded (FFPE) tissue samples (Li *et al.*, 2007).

To date, over 8600 miRNAs have been identified by traditional cloning techniques as well as bioinformatic efforts. They have been deposited in the online miRBase sequence database (Griffiths-Jones *et al.*, 2008; <http://microrna.sanger.ac.uk>; miRBase release 12.0), including currently more than 690 miRNA sequences for the human genome. Counts of miRNA entries in the miRBase as well as number of publications about miRNA research (Fig. 1) are permanently and rapidly growing, showing the evidence that miRNA research is one of the hottest and most important topics in the field right now.

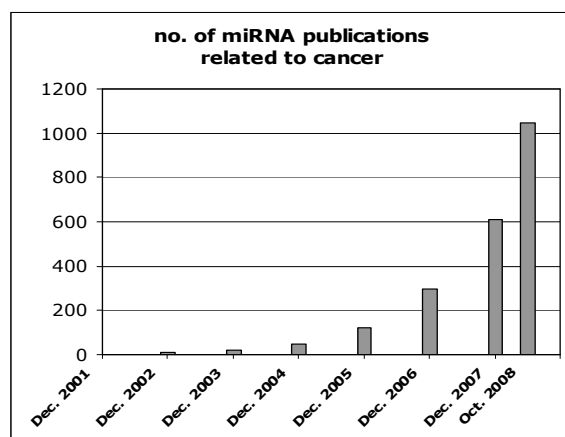


Figure1: Number of publications found in PubMed (<http://www.ncbi.nlm.nih.gov/pubmed/>) dealing with miRNA in combination with cancer starting with no publication in 2001 up to 1045 publications in October 2008.

Among closely related species, nearly all miRNAs are conserved, and they also have many homologs in distant species. In mammalian genomes, about 70% of miRNA genes are located in defined transcription units. They are often found in the introns of protein-coding genes and preferentially arranged in the same orientation as the predicted mRNA (Kim and Nam, 2006). Being then transcribed from the same promoter, the mRNA transcript and the miRNA usually have the same expression profiles. Alternatively, miRNA genes can be located in introns or exons of non-coding transcription units. The regulation of the tissue specific miRNA expression is not yet fully understood.

### Biogenesis and Molecular Functions

The first step within the biogenesis of miRNAs (Liu *et al.*, 2008) is the RNA polymerase II mediated transcription of long primary miRNA molecules, termed pri-miRNAs. The pri-miRNAs are cleaved within the nucleus by the so-called microprocessor, which consists mainly of the RNase III enzyme Drosha as well as a double-stranded RNA binding protein termed DGCR8/Pasha, resulting in ~70 nt stem loop intermediates, the precursor miRNAs (pre-miRNAs). After export to the cytoplasm, a double-stranded RNA of ~22 nt, the miRNA:miRNA\* duplex, is excised from the pre-miRNA hairpin by RNase III endonuclease Dicer. Afterwards, in a process referred to as miRNA loading, the miRNA:miRNA\* duplex is unwound and the mature miRNA binds to an Argonaute (Ago) protein. While in most cases the miRNA\* is degraded, the miRNA/Ago ribonucleoprotein forms the core component of the miRNP complex, which mediates miRNA function.

The miRNA-driven downregulation of gene expression occurs at the post-transcriptional level (Liu *et al.*, 2008). The miRNP complex interacts with specific mRNAs by pairing of nucleotide bases between the miRNA sequence and sequences in the 3'-untranslated region (3'UTR) of the mRNA and positioning of their bound Ago proteins onto mRNA targets. Depending on the extent of complementarity between the miRNA and the targeted mRNA, the induced translational repression either occurs by destabilisation or endonucleolytic cleavage of the mRNA. The exact molecular function is also dependent upon which Ago protein is attached.

In case of mRNA degradation, mature miRNAs bound to Ago2 pair with perfect complementary sequences in the target mRNA. While the miRNA remains intact, the mRNA is cleaved and decomposed subsequently via cellular pathways. While the target mRNA cleavage is in plants the predominant mechanism of regulation by miRNAs (Jones-Rhoades *et al.*, 2006), animal miRNAs tend to decrease target protein levels without endonucleolytically cleavage of the mRNA (Filipowicz *et al.*, 2005). In this case, the mature miRNA binds to only partially complementary sequences within the target mRNA. The subsequent repression of translation may occur during initiation, elongation or termination, but the detailed mechanism still needs to be elucidated (Wu and Belasco, 2008). Owing to their ability of target-binding with incomplete complementary, every single miRNA might bind to many different gene targets with diverse function and hence regulate a multiplicate number of genes. Detailed prediction of miRNA targets is still a challenge, but many bioinformatic approaches are using the first 2-8 nucleotides of the mature miRNA sequence as the so-called `miRNA-seed`.

Recently, Vasudevan *et al.* have shown that multiple miRNAs do not only repress translation, but can also enhance translation, depending on the cell cycle state.

#### **MicroRNAs play an important role in cancer**

Due to their function as gene regulators, miRNAs have been shown to control cell growth, differentiation and apoptosis. An impaired miRNA expression was found in many different human tumor types, not only for mature miRNAs but also for precursor miRNAs or even both. It is therefore evident that changes in miRNA expression levels might also play an important role in tumorigenesis. Though most miRNAs showed lower expression levels in tumors compared with normal tissues independent from the cell type, also overexpressed miRNAs have been found. In this respect, miRNA genes are thought to function as both tumor suppressors and oncogenes (Zhang *et al.*, 2007). For instance, the expression level of miRNA *let-7* has been shown to be reduced in lung cancer (Takamizawa *et al.*, 2004). In animals, the expression of *let-7* is dependent on developmental timing, with low levels in early stages of development and highest expression levels in

differentiated adult tissues. Downregulated expression of *let-7* results in loss of differentiation, which is a hallmark of cancerogenesis. Furthermore, it was demonstrated that the *RAS* oncogene is a direct target of miRNA *let-7*, suggesting that *let-7* works as a tumor suppressor gene in lung oncogenesis by its function as regulator of *RAS*. A second example is the downregulation of *miR-15* and *miR-16* in chronic lymphocytic leukemias, which target the antiapoptotic factor *BCL2*. On the other hand, oncogenic miRNAs promote cancer development by negatively inhibiting tumor suppressor genes or genes that regulate cell differentiation or apoptosis. An example is the *miR-17-92*, whose expression is significantly increased in several cancers like lung cancer and lymphomas and which emerges to enhance lung cancer cell growth. Another hint for the contribution of miRNAs in tumorigenesis is the fact that more than 50% of miRNA genes known so far are located in cancer-associated genomic regions (Calin *et al.*, 2004).

#### **Classification of cancer by studying miRNA expression**

Based on expression profiles of miRNAs, classification of tumors with respect to developmental origin as well as differentiation state became possible with a much higher accuracy than classification based on conventional mRNA expression studies (Lu *et al.*, 2005; Rosenfeld *et al.*, 2008). In a systematic study including multiple human cancers, nearly all analyzed miRNAs exhibited differential expression, enabling the discrimination of tumors of different developmental origin. This classification of human cancers was done by examining expression profiles of a relatively small number of about 200 miRNAs, while the study of expression profiles of about several thousand mRNA genes did not achieve the same result. Other studies showed a high correlation of miRNA expression with specific biopathologic features like tumor stage or proliferation index. In contrast to the evaluation of mRNA expression profiles which often do not have a direct biological consequence due to post-transcriptional modifications on the way from DNA to protein, miRNAs reflect more directly the functional level of the gene.

Another huge benefit of using miRNAs for the classification of cancer is their improved stability in

formalin-fixed paraffin-embedded (FFPE) tissues, which are routinely collected and archived in clinical research. While the longer mRNAs most likely undergo degradation and chemical modification during formalin fixation and processing, the tiny miRNAs, additionally protein protected by the RISC complex, outlast the fixation procedure as well as long term storage. An extensive comparison with snap frozen cells showed the reliability of using FFPE samples for achieving miRNA expression profiles (Li *et al.*, 2007).

#### **MicroRNAs as robust diagnostic and prognostic biomarkers**

These facts as well as their remarkable tissue specificity make miRNAs excellent biomarkers for the diagnosis and prognosis of cancer. Due to their gene regulation activities, the potential for using miRNA in cancer therapy is evident. So-called anti-miRNA oligonucleotides (AMOs), which are designed to be complementary to oncogenic miRNAs, are able to specifically inhibit miRNA activity in tumors. On the other hand, overexpression of miRNAs that act as tumor suppressors might also be beneficial for anticancer therapy.

MicroRNAs provide not only promising therapy approaches for cancer, but also for many other diseases in which they are involved as gene regulators. Recently, Umbach and colleagues (2008) found a remarkable participation of miRNAs to keep the herpes simplex virus 1 in a latent stage of infection. The inactive virus is inaccessible for any treatment, only if the herpes virus gives up latency and becomes active, it can be successfully medicated and hence be deleted. So, if the involved miRNAs can be blocked by using antisense reagents, the virus will be shifted into the active and therefor treatable state.

While the understanding for the gene regulation driven by miRNAs is under extensive research focus, the knowledge about the mechanisms regulating the gene expression of the miRNAs themselves still needs to be broadened. Amongst others, miRNAs are thought to be controlled by epigenetic mechanisms not only due to their tissue and tumor specific expression patterns. As a matter of fact, several miRNAs have shown to be regulated by DNA methylation. Treating human bladder cancer cells with demethylating agents,

Saito *et al.* have shown that ~5% of the human miRNAs became upregulated more than 3-fold. The strongest effect was seen for miR-127, whose corresponding gene was found to be embedded in a CpG island. After epigenetic reactivation of miR-127, one of its target genes, the proto-oncogene BCL6, became downregulated, leading to the assumption that miR-127 acts as a tumor suppressor gene. In cases like this, an epigenetic anticancer therapy becomes feasible.

#### **Microarray-based methods for miRNA expression profiling**

The most common method for high-throughput analysis of expression levels of miRNAs is the use of oligonucleotide-based microarrays (Liu, C.-G. *et al.*, 2004), enabling the measurement of differential gene expression of hundreds of miRNAs in a large number of samples simultaneously. Another microarray-based method, the so-called Microfluidic Primer Extension Assay (MPEA) based on the febit Geniom<sup>®</sup> microarray technology uses unlabeled miRNAs for hybridization on highly flexible microfluidic microarrays. In a second step, Klenow fragment of DNA polymerase I is added directly into the channels of the microfluidic chip, where the specific elongation of the bound miRNAs takes place. The method therefore combines in a double-staged way the specificity of a hybridization assay with the discriminatory power of an enzymatic extension (Vorwerk *et al.*, 2008).

The MPEA shows several outstanding advantages over any other existing microarray-based method. Since miRNA is used directly without preceding treatment like enrichment, PCR-based amplification or labeling, it is ensured that no experimental bias is introduced. Compared to the conventional hybridization assay, which is most suitable to discriminate mismatches in the central position of the hybridized target, MPEA provides an additional level of specificity due to the fact that the enzymatic elongation only can occur with nearly perfect matches at the 3'-end resulting in significantly less cross-hybridization signals. The MPEA also shows several key benefits compared to the conventional RNA-primed, array-based Klenow Extension (RAKE) assay (Nelson *et al.*, 2004). While the RAKE assay uses microarrays consisting of oligonucleotides bound with their 5'-end to the surface, the oligonucleotide capture probes of the

MPEA arrays are attached with their 3'-end to the surface. This fact does not only exclude self-elongation of the probes, but especially leads to exposition of the elongation reaction away from the surface to the lumen of the microchannel without any steric hindrance. A significantly lower amount of sample RNA is needed due to the use of microfluidic channels. The sensitivity is even high enough to reliably analyze nanogram amounts of total RNA coming from FFPE tissue samples, Fine Needle Aspiration or Laser Capture Microdissection without a need for amplification.

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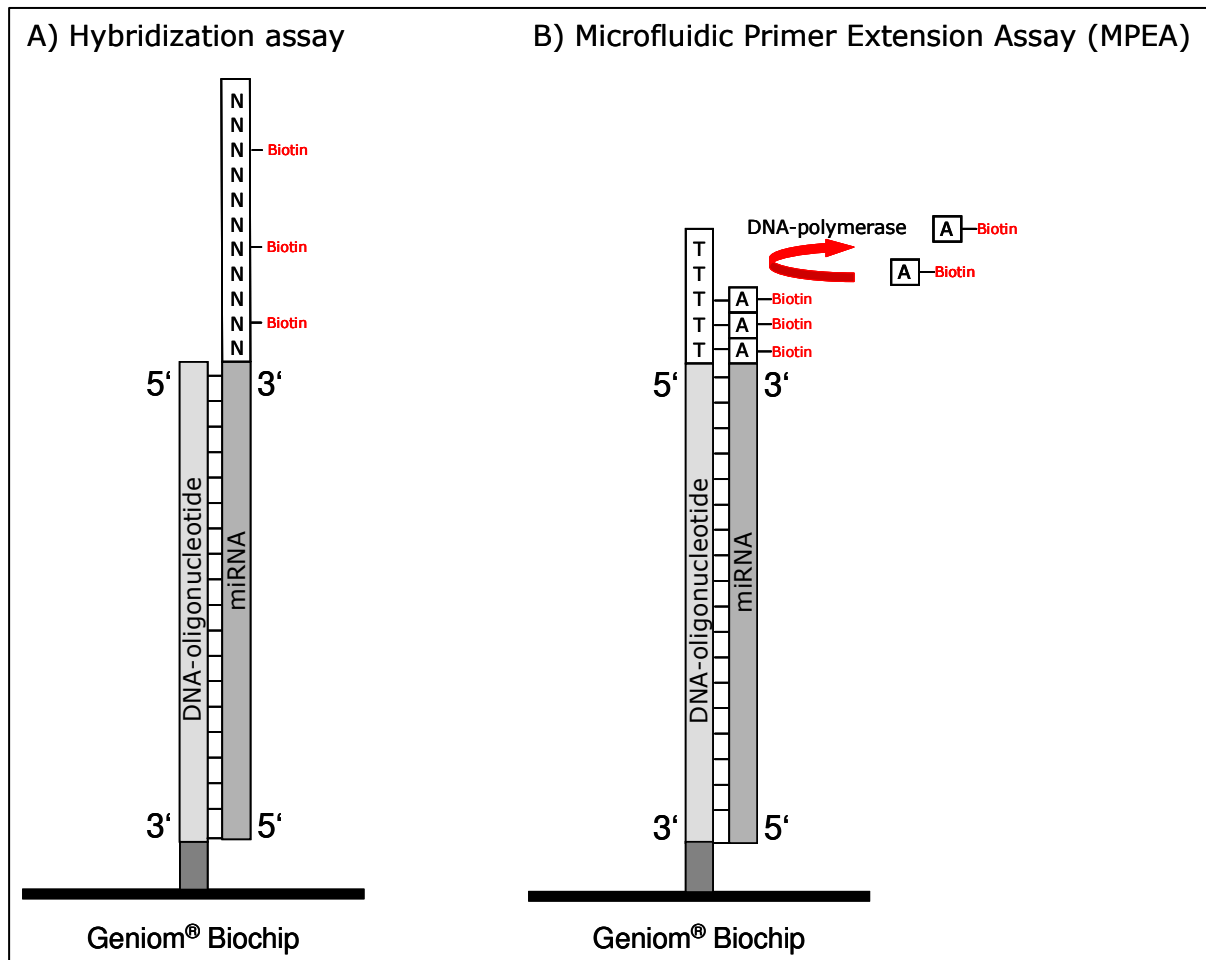


Figure 2: Comparison of two microarray-based assays used for miRNA profiling. In both cases, labeling was done using biotinylated nucleotides, which enables the subsequent staining with a streptavidin-phycoerythrin-conjugate. A): For the conventional hybridization assay, miRNAs are labeled first (here exemplarily shown using poly(A) tailing by the Ambion® mirVana™ miRNA Labeling Kit introducing a 20-50 tail of partially amine-modified nucleotides, which are then coupled to Biotin-XX-NHS-ester) and hybridized afterwards to the microarray containing reverse complementary oligonucleotides. B): Principle of the MPEA. The hybridized, unlabeled miRNA functions as a primer for an enzymatic elongation in which biotinylated nucleotides are incorporated.

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