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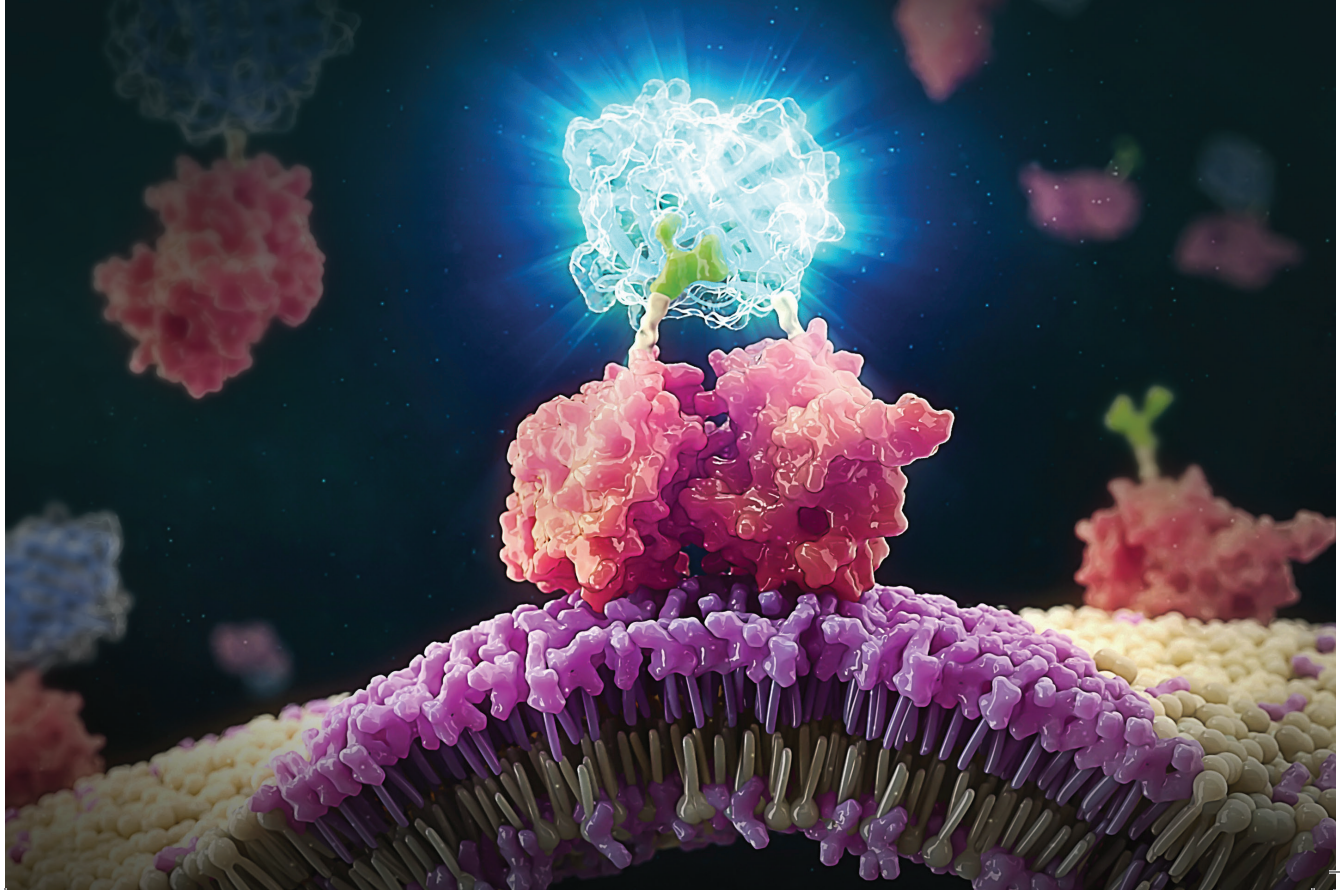
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# microRNAs and cancer

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**microRNAs (miRNAs) are small non-coding RNA molecules, 20-23 nucleotides long, involved in the regulation of gene expression. Recent studies have shown that miRNAs are implicated in human diseases, including cancer. They have been found to be differentially expressed in several malignancies compared to normal tissues and can affect the expression of oncogenes and tumor suppressors. The stability of these small RNAs and the development of more specific detection technologies make them also ideal candidate for the discovery of new cancer biomarkers.**

## Biology of cancer

Cancer is a common disease, with more than 30.000 cases reported in Finland in 2015 (Cancer Society of Finland, Cancer Registry). Although cancer mortality incidence has recently experienced a significant decrease in developed countries, the number of cancer cases continues to increase, due to aging populations and risk factor prevalence (Torre *et al.* 2015).

The biology of cancer is strictly linked to the control and regulation of cellular division. In normal conditions, every tissue needs to constantly regenerate by producing new cells at a constant and controlled rate, determined by the presence of specific molecular growth signals in the cellular environment (Biteau *et al.* 2011).

A disrupted control of the replication causes abnormal proliferation, independence of the cells from growth signals, evasion of programmed cell death (apoptosis), invasion to adjacent tissues and eventually spreading to distant sites in the body (metastasis). These features represent the classic hallmarks of cancer (Hanahan and Weinberg 2011).

The disruption of replication control is caused by genetic alterations. Mutations in the sequence or structure of the DNA lead to functional aberrations in the proteins encoded by genes affected by the mutational events and/or on their relative abundance in the affected cells (gene expression).

Mutations can be introduced by spontaneous errors of the DNA replication machinery or caused by exposure to DNA-damaging chemical or physical agents called mutagens (Tomasetti and Vogelstein 2015).

The classic model of cancer development contemplates mutations targeting specific types of coding genes termed oncogenes and tumor-suppressor genes.

Oncogenes encode for proteins that in normal conditions are responsible for initiating or sustaining cell proliferation when required, whereas tumor-suppressor genes have the opposite role of encoding for proteins that prevent cell proliferation, induce apoptosis and maintain genomic integrity and stability, by assuring that the genomic material of a cell is intact before committing to replication (Croce 2008).

Mutations in oncogenes lead to aberrant hyperactivation of proliferative factors and are therefore dominant. Mutations in tumor suppressor genes on the other hand are usually recessive, because one functional copy of these genes is generally sufficient to exert the correct function (Knudson 1971).

## microRNAs

The human genome encodes for approximately 20-25.000 genes, but these represent only about 2% of the total genomic content of human cells. The remaining 98% of the genome contains a large number of genes that do not encode for protein products, but exert their function in form of RNA transcripts (Alexander *et al.* 2010). These genes are therefore termed non-coding genes and their products are referred to as non-coding RNAs (ncRNAs). During the last two decades there has been increasing interest in studying the function of the non-coding part of the genome, as well as its potential role in the development of human diseases, in connection with the evolution of more sensitive and high-throughput technologies that allow large-scale profiling of these RNA molecules, such as mi-

croarrays and more recently next-generation sequencing (Esteller 2011).

microRNAs (miRNAs) are one of the better characterized classes of non-coding RNAs and are highly conserved in plants and animals. They have attracted considerable attention soon after the discovery of the first miRNA (lin-4) in *C. elegans* in 1993, which was found to influence the nematode development by negatively affecting the expression of the coding gene lin-14 and therefore revealing a new mechanism of regulation of gene expression (Lee *et al.* 1993).

miRNAs are small non-coding RNA molecules, 20-23 nucleotides long and are encoded by miRNA genes that can be mostly found in intergenic regions or in antisense orientation to coding genes. Some miRNA genes are also found within intronic regions of coding genes.

They are transcribed by the RNA polymerases II or III as double stranded precursors termed pri-miRNAs, that are subsequently processed into smaller pre-miRNAs and exported from the nucleus to the cytoplasm, where they are converted into single-stranded mature forms, associated with Ago proteins to form a RISC (RNA-induced silencing complex). The role of RISC is to direct the mature miRNAs to response elements consisting of complementary sequences that are usually located at the 3'- untranslated regions (3'- UTR) of target mRNAs (Gregory *et al.*, 2005). In humans and animals in general, the binding of the miRNA to the 3'- UTR of the mRNA causes interference with translation or target degradation and in consequence leads to a reduced expression of the protein encoded by the target gene (Bartel 2004), although exceptions of miRNAs that cause increased gene expression have been described (Truesdell *et al.* 2012).

The database of human miRNAs is constantly growing as new entries are being discovered and a current estimate shows about 2500 different miRNAs (Kozomara and Griffiths-Jones 2014).

As single miRNAs can potentially target up to hundreds of different coding genes and a single coding gene can also potentially be affected by several miRNAs, a very complex and intricate network emerges with these small molecules virtually affecting every biological function of the cells (Rajewsky 2006).

### The role of microRNAs in cancer

The consequence of the discovery of this additional layer of gene regulation was an increased focus of the research on the role of miRNAs in human diseases and especially in cancer. As mentioned above, a strictly controlled regulation of the expression of oncogenes and tumor-suppressor genes, responsible for cellular growth and homeostasis, is critical for the maintenance of a healthy tissue. Therefore, miRNAs that normally target tumor-suppressor genes or oncogenes can function themselves as oncogenic miRNAs or tumor-suppressive miRNAs respectively (Zhang *et al.*, 2007).

In the context of diseases, aberrant expression and/or mutations are commonly reported causes of altered miRNA function, but the subsequent identification and validation of miRNA target genes represent the crucial steps in understanding the molecular mechanisms of their actions in both physiological and pathological conditions. The identification of miRNA target genes is a particularly challenging task. Bioinformatic prediction provides invaluable help in the identification of putative target mRNAs and is based on computational algorithms that assess the complementarity between the sequence of the miRNAs and the 3'-UTRs of the mRNAs. These tools have provided substantial evidence for the complexity of the network of miRNA-target interactions (Rajewsky 2006). However, perfect complementarity between the miRNA and the 3'- UTR is not necessarily a reliable predictor of interaction and examples of miRNAs targeting mRNAs outside of their 3'-UTRs have also been recently described (Hausser and Zavolan 2014). *In vitro* approaches for miRNA identification include the use of cell lines transfected with synthetic miRNA mimic constructs or miRNA inhibitors. These are used to model miRNA over and under-expression respectively. Gene expression arrays or next-generation sequencing are used to study the expression of genes potentially affected by the resulting aberrant miRNA expression and therefore representing putative targets.

Both computational and *in vitro* approaches require further experimental validation to assess the actual interaction between the miRNAs of interest and the putative targets. The validation is performed using fluorescence-based reporter assays. The 3'-UTR of the putative target(s) is cloned downstream of a GFP or lu-

ciferase gene and the construct is co-transfected with the miRNA of interest into host cells. A mutated 3'-UTR construct is generally used as a control for the interaction and Western-blot/immunocytochemistry are further performed to assess the effect of the miRNAs of interest on the putative target(s) at protein level (Kuhn *et al.* 2009).

The first evidence of the role of miRNAs in human cancer was discovered in 2002 in chronic lymphocytic leukemia, where a chromosomal deletion at region 13q14 leads to the loss of two genes encoding for miR-15a and miR-16-1. These miRNAs normally target the anti-apoptotic factor Bcl2, reducing its expression and therefore increasing the rate of programmed cell-death over proliferation. miR-15a and 16-1 represent the very first example of tumor-suppressive miRNAs (Calin *et al.* 2002, Cimmino *et al.* 2005).

A well-studied example of oncogenic miRNA is the miR-17-92 cluster, which is also known as oncomiR-1. The cluster encodes for six different mature miRNA products, termed miR-17, 18a, 19a, 19b-1, 20a and 91-1, that are overexpressed in many human hematopoietic malignancies, such as B-cell lymphomas and Burkitt's lymphomas. These miRNAs can affect the expression of many target mRNAs, depending on the cellular context. Known important targets in cancer include the mRNAs for the tumor suppressor PTEN, the transcription factor E2F1 and the cyclin-dependent kinase inhibitor CDKN1A (He *et al.* 2005, Olive *et al.* 2010).

Several studies carried out using microarrays and next-generation sequencing technologies on both cell line models and clinical material have recently provided high-throughput global miRNA expression profiles in many human cancers, confirming that these small RNAs are deregulated in most human malignancies. Some recent reports have shown that the overall miRNA expression is significantly down-regulated in cancer compared with normal tissues (Lu *et al.* 2005). Moreover, hierarchical clustering of miRNA expression in tumors revealed that specific miRNA expression signatures can distinguish different tumor types, based on tissue of origin, as well as differentiate between cancerous and normal control tissues (Volinia *et al.* 2005, Lu *et al.* 2005). Specific miRNA signatures have also been found to be associated with more advanced tumor stages, highlighting the

potential of these small molecules as diagnostic and prognostic markers (Reddy 2015).

However, the results of these type of studies are often controversial, with the same miRNAs reported as over-expressed in some cases and under-expressed in others. This is due to several possible reasons, including sample quality and integrity, overall number of samples used, RNA collection methods, presence of contaminating cells and choice of reference control samples (Coppola *et al.* 2010).

Some challenges are also encountered in studies of single miRNA expression in cancer. When focusing the attention on a specific miRNA, qRT-PCR validation is usually performed using miRNA reverse-transcription and PCR probes. However, variation in the amount of starting material, sample preparation and RNA extraction, as well as in reverse transcription efficiency, can introduce errors. For this reason, a normalization of the raw expression values is usually performed using reference genes. Currently, there are no widely accepted general reference genes for miRNAs. Each reference gene should be carefully validated to be suitable for the type of samples used in each study.

In spite of the experimental challenges mentioned, the high stability of miRNAs and the increased efficiency in detection technologies make them promising candidate for the discovery of new cancer biomarkers. Researchers are now focusing on the examination of fluids such as plasma and urine to determine the levels of circulating miRNAs and to assess whether they can be used as minimally invasive markers of cancer onset or progression (Reddy, 2015). More retrospective and prospective studies involving large cohorts of clinical samples are still needed to confirm and validate the role of miRNAs in human cancer and to develop reliable miRNA-based assays for routine use in the clinics.

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