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Clinical and functional aspects of microRNA regulation in human cancers

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L’esistenza non è una corsa di 100 metri ma una straordinaria maratona
.....
Non dar retta a chi ti indica le scorciatoie, prova ad osare strade difficili, evita ciò che è comodo e diffida di chi te lo propone.

(PAOLO CREPET)

Cerca di essere felice, perché nonostante gli inganni e le amarezze,
la vita è meravigliosa.
.....
Ricorda che il giorno più bello della tua vita deve ancora arrivare, perciò vivi tranquillo e attendi fiducioso.

(R. BATTAGLIA)

Ai miei due cuccioli
Isabel e Claudia

ABSTRACT

miRNAs are short single-stranded non-coding RNAs which regulate gene expression at the post-transcriptional level in many biological processes, including proliferation, apoptosis and differentiation. A number of studies have shown the importance of miRNAs in carcinogenesis and accumulating evidence supports their role as diagnostic and prognostic biomarkers in human cancers. Moreover, dysregulation of miRNA processing factors, which are needed for miRNA maturation, have been recently shown to play an important role in tumor initiation and progression and to have a prognostic potential in different types of cancer. Despite all the achievements, our knowledge of the biological role of miRNAs and miRNA processing pathway in tumor development and progression is still in its infancy.

The general purpose of this thesis was to improve our understanding of the clinical and functional implications of miRNA deregulation in human cancer.

In **paper I**, we reported frequent deregulation of miRNA expression in lymph node metastases of malignant melanoma and melanoma cell lines as compared to normal melanocytes and showed its association to *BRAF* and *NRAS* mutational status. Moreover, we identified a two-miRNA signature that could predict survival in metastatic melanoma patients.

In **paper II**, we performed genome-wide miRNA expression profiling of adrenocortical tumors and identified distinct miRNA expression patterns in adrenocortical carcinomas (ACC) compared to adenomas and normal adrenal cortices. Over-expression of *miR-483-3p/-5p* and down-regulation of *miR-195* and *miR-497* were the most common features of ACC. We also elucidated the functional consequences of deregulation of these 4 miRNAs on cell proliferation and apoptosis in ACC cells and, in addition, we demonstrated the potential involvement of the pro-apoptotic factor PUMA (a target of *miR-483-3p*) in adrenocortical tumors. Moreover, we found novel miRNAs associated with short survival in ACC.

In **paper III**, we evaluated the expression and the potential role of the main components of the miRNA machinery in adrenocortical tumors. We observed frequent over-expression of TRBP2 in ACC and found that *TRBP2* mRNA expression level is a reliable predictor of carcinoma among adrenocortical tumors. These data suggest that TRBP2 may be a novel and sensitive biomarker for adrenocortical tumors. Functionally, we unraveled the potential oncogenic role of TRBP2 in ACC and identified some of the molecular mechanisms involved in the regulation of TRBP2 expression in this tumor type.

In **paper IV**, we analyzed the expression of miRNAs and miRNA machinery factors in diffuse large B-cell lymphoma (DLBCL). We identified miRNA signatures that could discriminate DLBCL tumors from non-neoplastic tissue and found subsets of miRNAs able to classify DLBCL sub-types. Moreover, we showed dysregulation of miRNA machinery factors in DLBCL and demonstrated that it could influence miRNA processing. We also showed, similarly to our observations in ACC, that deregulation of TRBP2 expression could affect cell proliferation and cell death in lymphoma cell lines, suggesting its potential oncogenic role in DLBCL development.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals throughout the text.

I. **Caramuta S***, Egyházi S, Rodolfo M, Witten D, Hansson J, Larsson C, Lui WO*****.
MicroRNA expression profiles associated with mutational status and survival in malignant melanoma.
J Invest Dermatol 2010 Aug;130 (8):2062-70.

II. Özata DM[#], **Caramuta S*[#]**, Velázquez-Fernandez D, Akçakaya P, Xie H, Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO*****.
The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma.
Endocr Relat Cancer 2011 Oct;18 (6):643-55.

III. **Caramuta S***, Lee L, Özata DM, Akçakaya P, Xie H, Höög A, Zedenius J, Bäckdahl M, Larsson C, Lui WO*****.
Clinical and functional impact of TRBP2 over-expression in adrenocortical carcinoma.
Manuscript.

IV. **Caramuta S***, Berglund M, Lee L, Özata DM, Akçakaya P, Georgii-Hemming P, Xie H, Amini RM, Enblad G, Larsson C, Lui WO*****.
Role of microRNAs and microRNA machinery in the pathogenesis of diffuse large B-cell lymphomas.
Manuscript.

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RELATED PUBLICATIONS

1. Akçakaya P, Ekelund S, Kolosenko I, **Caramuta S**, Özata DM, Xie H, Lindforss U, Olivecrona H, Lui WO.
miR-185 and *miR-133b* deregulation is associated with overall survival and metastasis in colorectal cancer.
Int J Oncol 2011 Aug;39 (2):311-8

2. Xie H, Zhao Y, **Caramuta S**, Larsson C, Lui WO.
miR-205 expression promotes cell proliferation and migration of human cervical cancer cells.
Submitted manuscript.

CONTENTS

LIST OF ABBREVIATIONS

A	Adenosine
ABC	Activated B cell-like
ACC	Adrenocortical carcinoma
ADAR	Adenosine deaminase acting on RNA
Ago	Argonaute
AIB1	Amplified in breast cancer gene 1
AML	Acute myeloid leukemia
ATCC	American Type Culture Collection
AVV	Adenovirus-associated vector
BBC3/PUMA	Bcl-2 binding component 3/PUMA
BCL2	B-cell lymphoma 2
BCL6	B-cell lymphoma 6
BIC	B-cell integration cluster
BIM	Bcl2-ineracting mediator of cell death
C	Cytosine
CCND1	Cyclin D1
cDNA	Complementary DNA
c/EBPbeta	CCAAT enhancer-binding protein beta
CLIP	Cross-linking combined to immunoprecipitation
CLL	Chronic lymphatic leukemia
DCL1	DICER-LIKE 1
ddNTP	Dideoxynucleotide triphosphate
DGCR8	DiGeorge Syndrome critical Region 8
DIG	Digoxygenin
DLBCL	Diffuse large B-cell lymphoma
DLG1	Disks large homolog 1
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
ERα	Estrogen receptor α
FDR	False discovery rate
G	Guanosine
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GCB	Germinal center B cell-like
HEN	ENHANCER 1
HIF	Hypoxia-inducible factor
HOTAIR	HOX transcript antisense RNA
HYL1	HYPONASTIC LEAVES 1
I	Inosine
IGF-2	Insulin-like growth factor 2
IHC	Immunohistochemistry
IP	Immunoprecipitation
IRF-4	Interferon regulatory factor 4
LINE-1	Long interspersed nuclear element type 1
LNA	Locked nucleic acid

LN	Lymph node
MAPK	Mitogen-activated protein kinase
MDS	Myelodysplastic syndrome
miRNA	microRNA
miRISC	miRNA-induced silencing complex
MGB	Minor groove binder
mRNA	messanger RNA
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ncRNA	Non-coding RNA
NFQ	Non-fluorescent quencher
NTP	Nucleotide triphosphate
2'OMe	2'-O-methyl
2'OME	2'-O-methoxyethyl
ORF	Open reading frame
PACT	Protein activator of PKR
PAD	PAP associated domain
PAM	Prediction Analysis of Microarrays
PINK1	PTEN-induced putative kinase 1
piRNA	Piwi-interacting RNA
pNA	Chromophore p-nitroaniline
Pre-miRNA	Precursor-miRNA
Pri-miRNA	Primary-miRNA
PTEN	Phosphatase and tensin homolog
qRT-PCR	Quantitative real time – polymerase chain reaction
RNA	Ribonucleic acid
RNU6B	U6B small nuclear RNA
SAM	Significance Analysis of Microarrays
SE	SERRATE
SHIP	Src-homology 2 domain-containing inositol-6-phosphatase
siRNA	Small interfering RNA
SNP	Single nucleotide polymorphism
snRNA	Small nuclear RNA
snoRNA	Small nucleolar RNA
svRNA	Small vault RNA
TIMP3	Metallo-proteinase 3
TUDOR-SN	Tudor staphylococcal nuclease
TP53	Tumor protein 53
TRBP	TAR RNA-binding protein
tUCR	Transcribed ultraconserved region
TUT1	Terminal uridylyl tranferase 1
UV	Ultraviolet light
UTR	Untranslated region
vi-miRNA	Virus-encoded miRNAs
WNT3A	Wingless-type MMTV integration site family, member 3A
WST-1	4-(3-(4-iodophenyl)-2-(4 nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate
XPO5	Exportin 5
ZCCHC	Zinc finger CCHC domain

1 INTRODUCTION

The central dogma of molecular biology proposed by Francis Crick in 1958 (Crick, 1958) is based on the assumption that genes are transcribed to RNA and subsequently translated into proteins which play a major role in most of the regulatory molecular pathways from unicellular to complex organisms. However, in the last decade, the development of high-throughput sequencing platforms has led the scientific community to the discovery that non-protein-coding RNAs (ncRNAs) represent over 90% of the human genome (Bertone et al., 2004; Birney et al., 2007). At the present, many classes of ncRNAs have been identified and, although, the function is not yet completely uncovered their role in regulatory cellular pathways is well recognized. Non-coding RNAs can be classified as small non-coding RNAs (18-200nt) which include *e.g.* microRNAs (miRNAs), small interfering RNAs (siRNAs), piwi-interacting RNAs (piRNAs), small vault RNAs (svRNAs) small nuclear RNAs (snRNAs), vault RNAs (vtRNAs), small nucleolar RNAs (snoRNAs) (Aravin et al., 2006; Bartel, 2004; Kawaji et al., 2008) or long non-coding RNAs (200nt-100kb) including among others long intergenic ncRNAs (such as *HOTAIR*) and transcribed ultraconserved regions (tUCRS) (Calin et al., 2007; Rinn et al., 2007).

In this thesis, we will focus on miRNAs, which probably represent the most studied class of ncRNAs, with special emphasis on their role in tumor development and progression and the clinical impact on diagnosis, prognosis and treatment of human malignancies.

1.1 MiRNA discovery

miRNAs are single-stranded RNAs of ~22 nucleotides in length that are generated by the RNase-III type enzyme Dicer from endogenous hairpin-shaped transcripts and regulate gene expression at the post-transcriptional level (Ambros, 2001; Kim, 2005). The first discovery in the miRNA field dates back to 1993, when Victor Ambros and collaborators cloned *lin-4*, a gene involved in *C. elegans* development regulation, that does not encode for any protein but produces a ~22nt long RNA molecule from a longer hairpin-shaped RNA precursor (Lee et al., 1993; Wightman et al., 1993). Further, they and others discovered that the *lin-4* RNA acted as an antisense translational repressor and could regulate expression of the *lin-14* gene binding to complementary sequences in its 3'untranslated region (UTR) (Lee et al., 1993; Wightman et al., 1993). *Lin-4* was believed to be a unique species until year 2000 when a new small RNA, *let-7,* was reported in *C. elegans* (Reinhart et al., 2000).

Interestingly, *let-7* was also found to be highly conserved in a variety of organisms (Pasquinelli et al., 2000). Since then a large number of similar small RNA sequences, for the first time termed as miRNAs in 2001, were discovered in a wide range of organism from nematodes to vertebrates, plants and viruses. miRNAs are now recognized as a conserved class of small non-coding RNAs which are believed to regulate more than 60% of the human protein-coding genes (Friedman et al., 2009) and are involved in many biological processes such as development, proliferation, differentiation, antiviral defense, and tumorigenesis (Ambros, 2001; Bartel, 2004; Bennasser et al., 2004; He and Hannon, 2004; Kim, 2005). Until November 2011, nearly 1500 human mature miRNAs have been annotated in the miRBase database Release 18.0 (http://www.mirbase.org/; Griffiths-Jones, 2004).

1.2 MiRNA biogenesis and function

The majority of known miRNAs are located within introns or exons of protein coding or noncoding genes. Nearly 50% derive from non-coding RNA transcripts while only 10% have been found to be transcribed from exons of either protein-coding or non-coding transcription units. The other miRNAs are located within introns of protein-coding genes and are often co-transcribed together with their host transcripts. Approximately 25-30% of intronic miRNAs are instead transcribed from independent promoters. A second group of miRNAs are located in intergenic regions and derive from independent transcription units (Corcoran et al., 2009; Rodriguez et al., 2004). Moreover, over 40% of human miRNAs are found as clusters and are co-transcribed from a single polycistronic transcription unit (Altuvia et al., 2005). Interestingly, in some cases, miRNA can be transcribed from two different genes (e.g. *miR-29b-1* from chromosome 7 and *miR-29b-2* from chromosome 1. Both of these miRNAs have the same mature sequence (Garzon et al., 2009).

1.2.1 Biogenesis: transcription and miRNA maturation

miRNA biogenesis is a complex multistep process. In the canonical pathway, miRNAs are transcribed as long primary transcripts (pri-miRNA) mostly by RNA polymerase II (Lee et al., 2004), although polymerase III is also required for the transcription of a subset of miRNAs (Borchert et al., 2006). The pri-miRNAs are several kilobases long transcripts characterized by a stem-loop structure and are cleaved within the nucleus by the RNase III-type enzyme Drosha, and its co-factor DGCR8 (DiGeorge Syndrome Critical Region 8), to release the precursor miRNAs (Pre-miRNA) (Lee et al., 2003). The 60-70nt long hairpin-like pre-miRNAs are exported to the cytoplasm through exportin 5 (XPO5) (Bohnsack et al., 2004) and further processed by Dicer (another RNase III-type enzyme), together with the co-factors TRBP (TAR RNA-binding

protein) and PACT (protein activator of PKR), to form a ~22nt asymmetric miRNA duplex (Hutvagner et al., 2001; Ketting et al., 2001; Lee et al., 2006). The miRNA duplex is a short-lived intermediate which is loaded into the miRNA-induced silencing complex (miRISC), where argonaute (Ago) proteins are the core effector components (Filipowicz et al., 2005). In the miRISC complex one strand of the duplex becomes the mature miRNAs while the other strand is degraded depending on the thermodynamic stability of the two ends of the duplex (Khvorova et al., 2003; Schwarz et al., 2003).

1.2.1.1 Alternative pathways and regulatory mechanisms in miRNA biogenesis

Although the miRNA processing pathway was previously thought to be a linear and universal process, alternative biogenesis pathways have recently been described. Mirtrons are short introns that after splicing resemble the pre-miRNA hairpin structure and enter the miRNA biogenesis process and bypass the Drosha-mediated cleavage (Berezikov et al., 2007). Another non-canonical biogenesis pathway is represented by Dicer-independent miRNAs: pre-miRNA is directly incorporated in the Ago complexes where the miRNA maturation is mediated by Ago cleavage activity producing an intermediate product which is further processed by endo- or exonucleases (Cheloufi et al., 2010). In addition, post-transcriptional regulation processes that generate mature miRNA variants (or isomiRs) have been reported. The pri-miRNA transcripts can be altered through nuclear RNA editing by the enzyme ADAR (adenosine deaminase acting on RNA) which catalyzes the conversion from adenosine (A) to inosine (I). Changing the sequence of pri-miRNAs, the RNA editing may affect base-pairing and structural properties of the primary transcripts and can influence their further processing as well as the function of the mature miRNA (altering target specificity through sequence changing in the seed region) (Blow et al., 2006; Kawahara et al., 2008). For example, the editing of *miR-142* modifies the secondary structure of the pri-miRNA and prevents the cleavage by Drosha, leading to degradation of *pri-miR-142* by the ribonuclease TUDOR-SN (Tudor staphylococcal nuclease) (Yang et al., 2006b). RNA editing can also occur on the pre-miR species and can prevent Dicer processing. This results in an accumulation of the pre-miR affecting the expression of the mature form (Kawahara et al., 2008). The isomer variability can also be generated by a shift of Dicer or Drosha cleavage position at the 5' or 3'-end or by nucleotide additions at the 3'-end. Modifications at the 3'-end are common post-transcriptional events that are phylogenetically conserved and are mediated by the activity of multiple nucleotidyltransferases. These modifications result predominantly from adenylation or uridylation although addition of guanine has also been observed. Uridylation, at least in part, seems to be catalyzed by ZCCHC11 (zinc finger CCHC domain containing 11), ZCCHC6 (zinc finger CCHC domain containing 11) and TUT1 (terminal uridylyl transferase 1) nucleotidyltransferase and primarily take place on the pre-miRNA and

affects the processing by Dicer. Adenylation is mediated by PAPD4 and PAPD5 (PAP associated domain containing 4 or 5) enzymes after Dicer cleavage. All these modifications are related with reduced miRNA efficiency in target recognition (Burroughs et al., 2010; Morin et al., 2008; Wyman et al., 2011).

1.2.2 Function: target recognition and regulation

The mature miRNA guides the miRISC to specifically recognize and regulate particular mRNA targets. The miRNA binding sites are generally present in the 3'UTR of the target transcripts: location, number and proximity of miRNA binding sites along an mRNA target contribute to the miRNA-mediated gene regulation (Brennecke et al., 2005; Grimson et al., 2007; Lewis et al., 2005; Nielsen et al., 2007). The most stringent requirement for miRNA-mRNA pairing is the perfect interaction of miRNA nucleotides 2-7 to the 5' end, representing the seed region. Pairing of the 3' region (3'-supplementary sites and 3'-compensatory sites) of the miRNA can enhance target recognition and in some cases can even compensate for a mismatch to the seed region (Bartel, 2009). Recently, "centered sites" have been described to indicate miRNA target sites lacking of both perfect seed pairing and 3'-compensatory sites but instead presenting 11-12 contiguous perfect pairs to the center of the miRNA (Shin et al., 2010). The degree of miRNA-mRNA complementarity will define the outcome of this interaction. Perfect base pairing, either in the coding region or in the 3'UTR of the target, will lead to miRNA-dependent cleavage of mRNA-target (Meister et al., 2004; Yekta et al., 2004). An imperfect miRNA-mRNA target pairing will cause repression of translation and/or mRNA deadenylation, followed by destabilization of the targets (Pillai et al., 2007).

As previously described, post-transcriptional modifications resulting in changes of the mature miRNA sequence can influence the miRNA-mRNA target interaction and regulation. In addition, SNPs in the 3'UTR of the target genes might modify binding sites altering miRNA function. Single nucleotide polymorphism has been observed within the binding site recognized by *let-7* in the 3'UTR of *KRAS* and was associated with an increased risk of non-small cell lung cancer (Chin et al., 2008). Recently, Sandberg *et al.* showed that highly proliferating cells express mRNAs with shorter 3'UTRs. This mechanism leads to a reduction of miRNA binding sites in the 3'UTR and consequently proliferating cells become resistant to miRNA-dependent repression (Sandberg et al., 2008). Alternatively, mRNA binding factors, under certain conditions (*e.g.* cellular stress), may associate with miRNA binding sites in the 3'UTR of specific mRNA target and affect miRNA-guided gene silencing (Bhattacharyya et al., 2006; Kedde et al., 2007).

Recently, non-canonical miRNA-mediated mechanisms of mRNA target regulation have been proposed. Some miRNAs were shown to bind to the 5'UTR of target genes and exceptionally to activate rather than repress gene expression (Lytle et al., 2007; Orom et al., 2008). Interestingly, under specific conditions, miRNAs can also switch from repression to translational activation of their own targets. Vasudevan *et al*. showed that human Ago2 activates translation of target mRNAs during cell cycle arrest caused by serum starvation or contact inhibition while, in proliferating cells, the same targets are normally repressed (Vasudevan et al., 2007). Moreover, miRNAs can bind to ribonucleoproteins in a RISC-independent manner and interfere with their RNA binding properties (decoy activity) (Eiring et al., 2010). Additional studies have shown that miRNAs can regulate gene expression also at the transcriptional level by binding directly to the DNA (Khraiwesh et al., 2010; Kim et al., 2008) (Figure 1).

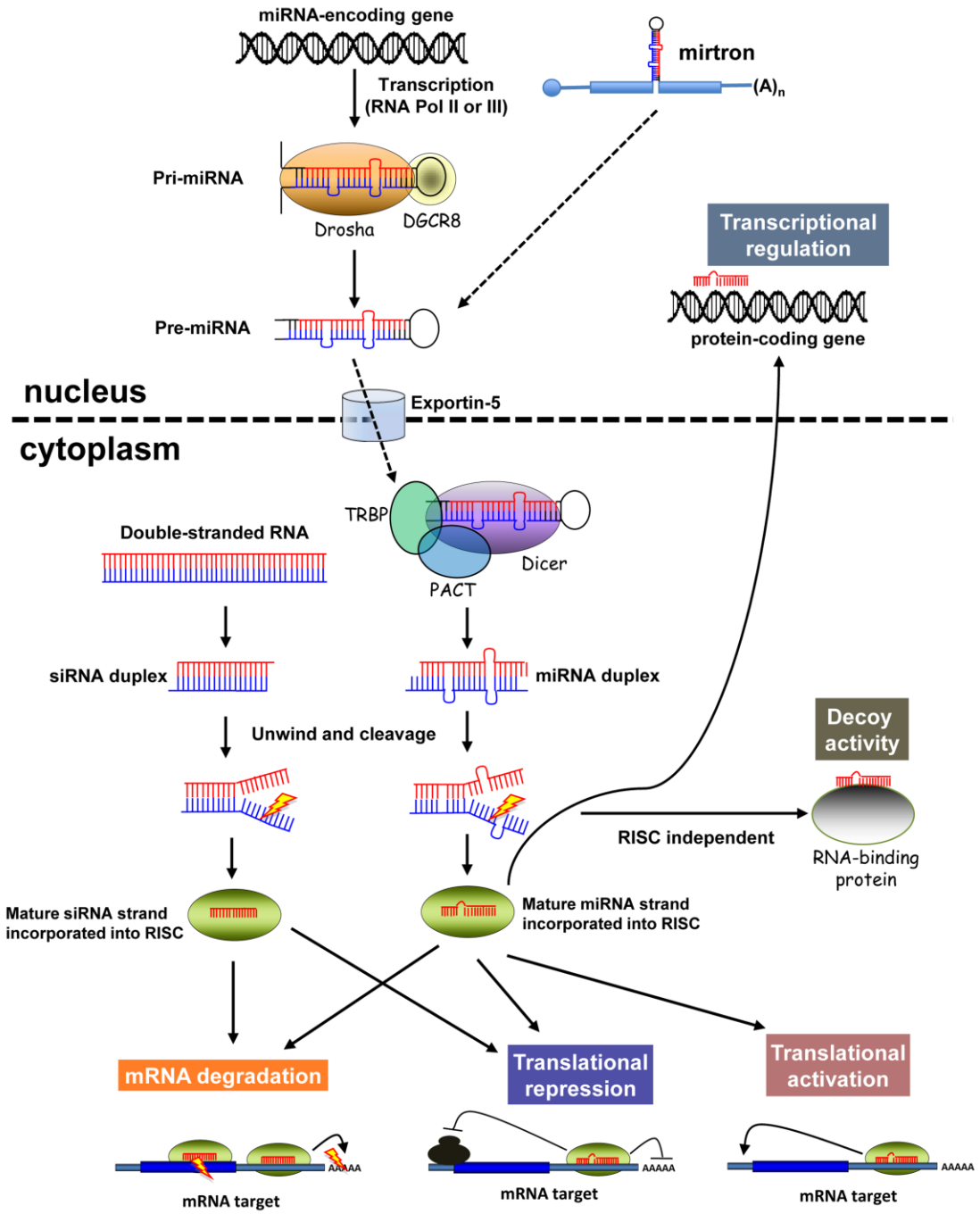


Figure 1. Biogenesis and function of miRNAs and siRNAs.

1.2.3 Not only metazoan miRNAs

It was generally believed that miRNAs belong to a class of small non-coding RNAs regulating gene expression only in metazoans. However, later in 2002, cloning of small RNAs revealed the presence of miRNAs also in plants, indicating that miRNAs arose early in eukaryotic evolution. Like in metazoans, miRNAs in plants are endogenously expressed 20-24nt long non-coding RNAs which derive from hairpin-shaped precursors and generally are evolutionary conserved (Reinhart et al., 2002). At present more than 4000 miRNAs from 52 plant species have been annotated in the miRBase database (Griffiths-Jones, 2004). Differences between plant and animal miRNAs are mainly related to their biogenesis. In plants, miRNA genes are primarily located in genomic regions not associated with protein-coding genes indicating that they are produced from their own transcriptional units (Reinhart et al., 2002). Like in metazoans miRNA genes are transcribed by RNA polymerase II into long primary transcripts; however, the processing of pri-miRNA into an asymmetric miRNA duplex occurs entirely in the nucleus and is carried out by a RNaseIII enzyme, DCL1 (DICER-LIKE 1). Two co-factors, HYL1 (HYPONASTIC LEAVES 1, dsRNA binding protein) and SE (SERRATE, C2H2-type zinc finger), are also required for pri-miRNA processing (Han et al., 2004; Yang et al., 2006a). Different from animals pri-miRNAs which have a ~70nt stem-loop structure, in plants the stem-loop structure of pri-miRNA can be greatly variable in length and is usually larger than those in animals (>100nt) (Reinhart et al., 2002). After DCL-mediated cleavage and HEN1 (ENHANCER 1) methylation (Yu et al., 2005), the single stranded miRNA is exported to the cytoplasm and incorporated in the RISC complex similarly to metazoan miRNAs. Plant miRNAs show a better complementarity to their targets compared to miRNAs in animal and generally induce cleavage of the targeted transcript (Reinhart et al., 2002; Rhoades et al., 2002) although cases of gene expression regulation via translational repression have been reported (Chen, 2004). In plants, miRNAs can regulate a diverse number of pathways including development, cell differentiation, hormone signaling and stress response (Allen et al., 2005; Reinhart et al., 2002).

Virus-encoded miRNAs (vi-miRNAs) were identified for the first time in Epstein-Barr virus (EBV) by Pfeffer and colleagues in 2004 (Pfeffer et al., 2004). Today, more than 200 virus-encoded miRNAs have been identified (miRBase database Release 18.0; Griffiths-Jones, 2004) in DNA viruses and retroviruses but not in RNA viruses. Given that miRNA biogenesis is a process that is initiated in the nucleus, it is believed that in viruses with RNA genome, which replicate exclusively in the cytoplasm of the infected cells, the generation of miRNAs is not available (Pfeffer and Voinnet, 2006). Although natural miRNAs encoded by RNA viruses have not yet be identified, a recent study showed that, at least at a mechanistic level, the production of mature miRNAs is

possible also for Tick-Borne Encephalitis Virus, an ssRNA virus (Rouha et al., 2010). However, data showing production of miRNAs in other RNA viruses are not available yet. Today, the function of most of the viral miRNAs is still unknown, however it has been shown that they can target and regulate the expression of both host genes, which may interfere with stages in the viral life cycle, and virally encoded transcripts (Pfeffer et al., 2004). The latter mechanism seems to be relevant to regulate the accumulation of viral products required during specific phases of infection (Pfeffer and Voinnet, 2006).

1.2.4 siRNAs and miRNAs: similarities and differences

The RNA interference (RNAi) pathway is an evolutionary conserved mechanism among eukaryotes involved in sequence-specific gene silencing. This biological process, which was originally observed in plants in the early '90s and later in the worm *C. elegans*, is triggered by long double-stranded RNA that either is artificially introduced in cells or is naturally generated, such as sense and antisense transcripts, transposons or intermediate products of viral replication (Cogoni and Macino, 1999; Fire et al., 1998; Ketting et al., 1999; Li et al., 2002; Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990). Although, siRNAs and miRNAs differ in their molecular origins, they share many common features in terms of molecular characteristics, biogenesis and target recognition modalities. Like miRNAs, the double-stranded RNA is processed in the cytoplasm by Dicer into ~22nt double-stranded small interfering RNAs (siRNAs). However, during miRNA biogenesis each pre-miRNA generates a single miRNA duplex, while a multitude of siRNA molecules are produced from both strands of each dsRNA precursor (Hamilton and Baulcombe, 1999; Ketting et al., 2001; Parrish et al., 2000). Subsequently, siRNAs are incorporated in the RISC complex as single-stranded RNAs and can bind to their target with perfect complementarity and therefore regulate gene expression by directing cleavage of the target mRNAs (Elbashir et al., 2001; Martinez et al., 2002). On the other hand, when siRNA pair imperfectly to their targets, they can act as miRNAs and induce translational repression rather than mRNA cleavage (Doench et al., 2003) (Figure 1). While the miRNA-mediated gene regulation is referred to as heterosilencing (the regulated genes are distinct from the gene that the miRNAs originate from), siRNAs are defined as *cis*-acting since they induce the degradation of RNAs from which they derive (*e.g.* transposons or viral RNAs) (Bartel, 2004).

Primarily, it seems that RNA interference functions as a defense mechanism against viral infection in flies and plants, although viruses have themselves evolved strategies to suppress the RNA-silencing pathway (Baulcombe, 2004; Keene et al., 2004; Li et al., 2002). However, it is not clear whether RNAi acts as antiviral defense mechanisms also in mammalian cells. In these organisms the presence of viral duplexes activates an interferon-mediated pathway resulting in non-specific RNA degradation and repression

of protein synthesis which will lead to destruction of virus-infected cells by apoptosis (Katze et al., 2002). Although, these observations imply that RNAi-mediated antiviral response is not needed in mammals, several recent studies indicate that mammalian viruses can interact and suppress the cellular RNAi machinery, suggesting that viruses infecting mammalian cells are targeted by the RNAi machinery and that RNAi might regulate viral replication (Johnson et al., 2004; Li et al., 2004; Sullivan and Ganem, 2005). Furthermore, virus-derived siRNAs have recently been identified, suggesting that viral sequences can be processed by Dicer (Andersson et al., 2005; Lu and Cullen, 2004). Adenoviral virus-associated RNA I and II are expressed at high levels during infection by adenovirus and are responsible of blocking the activation of PKR, which is involved in cellular antiviral defense mechanisms. In addition, they can also inhibit the RNAi pathway in the infected host cells: the virus-associated RNAs are processed by Dicer and the derived siRNAs are incorporated in the RISC. However, at the moment, their modality of action is still unclear. It has been proposed that they might act by saturating the RNAi machinery preventing to take action against viral coding genes. Alternatively, the virus associated-siRNAs might work as miRNAs and silence gene expression in the host (Andersson et al., 2005; Lu and Cullen, 2004).

It has been shown that endogenous siRNAs can silence retrotrasposons in plants (Hamilton et al., 2002) and more recently natural siRNAs, derived from LINE-1 (Long interspersed nuclear element type 1, or L1) retrotrasposons, have been identified in cultured mammalian cells (Watanabe et al., 2006). L1 retrotrasposons are highly abundant in human comprising approximately 17% of the human genome, and can be transcribed from sense and antisense promoters. The resulting bidirectional transcripts can be processed to siRNAs that suppress retrotransposition of L1 elements by RNAi mechanism (Yang and Kazazian, 2006). Endogenous siRNAs derived from transposable elements in mammals have also been identified in fully grown mouse oocytes (Watanabe et al., 2006).

In addition, there are evidences showing that the RNAi pathway, beside post-transcriptional gene regulation, is also involved in gene silencing at the transcriptional level. This mechanism, known as siRNA-directed transcriptional silencing (RdTS), was initially reported in plants and yeasts where siRNAs can induce specific promoter methylation or histone and heterochromatin modifications to repress gene expression (Grewal and Moazed, 2003; Mette et al., 2000; Volpe et al., 2002). Later, RdTS was also found in mammalian cells, indicating an evolutionary conservation, and was shown to involve epigenetic changes, similar to plants and yeast (Morris et al., 2004; Ting et al., 2005; Weinberg et al., 2006). Although the processes involved in siRNA-directed transcriptional silencing in mammals are still poorly understood, different possible mechanisms have been proposed. A few studies demonstrated the

transcriptional silencing might require the involvement of Argonaute proteins (such as AGO1 and AGO2) (Janowski et al., 2006) or alternatively siRNAs might bind to single-stranded DNA regions, *e.g.* transcription start sites, and physically inhibit the transcription (Janowski et al., 2005). Recently, it has also been shown that siRNAs bind to single-stranded promoter-associated RNAs forming RNA:RNA hybrids which function as recognition sites to direct gene silencing complexes (Han et al., 2007).

1.3 MiRNA and cancer

Tumor development and progression is generally regarded as a multistep process characterized by accumulation of genetic changes over time. Each change contributes to the accumulation of specific features that eventually will lead to the malignant status. Malignant cancer cells grow in an uncontrolled manner, become resistant to programmed cell death (apoptosis) and acquire the ability to invade adjacent tissues and to metastasize.

A potential involvement of miRNAs in pathogenesis of cancer was shown for the first time in 2002, when Calin and colleagues (Calin et al., 2002) identified two miRNAs, *miR-15a* and *miR-16* which are located in chromosomal region 13q14. This genomic region is deleted in most cases of chronic lymphocytic leukemia (CLL) and the two miRNAs were under-expressed in almost 70% of the cases carrying this deletion (Calin et al., 2002). In a subsequent study, the same group demonstrated that the majority of human miRNAs are located in genomic regions associated with chromosomal aberrations in human cancers (Calin et al., 2004b). Further, Zhang *et al.* showed that a high proportion of genomic loci containing miRNA genes presented DNA copy number variations (Zhang et al., 2006). After these early studies, several groups performed miRNA expression profiling and described abnormal miRNA expression as a frequent event in a variety of different cancer types, including solid and hematological tumors (Calin et al., 2006; Calin et al., 2004a; Lu et al., 2005).

1.3.1 MiRNAs as oncogenes or tumor suppressor genes

Similar to protein-coding genes, over-expressed or down-regulated miRNAs in cancer are believed to act either as oncogenes or tumor-suppressor genes, respectively (Figure 2). However it may be inappropriate to define a single miRNA as either an oncogene or a tumor suppressor since the function of each miRNA might depend on the cellular context in which it acts.

1.3.1.1 Causes of miRNA deregulation

The molecular processes involved in miRNA deregulation in human cancer are not clearly understood, however several of evidences indicate that multiple genetic mechanisms might be involved. Given their location in fragile sites of the human genome, miRNA alteration in cancer may be caused, for instance, by chromosomal aberrations such as amplification, translocation, deletion or insertion (Calin et al., 2002; Gauwerky et al., 1989; He et al., 2005).

In addition to these mechanisms, it has been proposed that transcription factors can be involved in miRNA dysregulation. The tumor suppressor p53 can directly induce the expression of *miR-34a* by binding to the promoter region of the gene that generates *miR-34a*. This miRNA might be a mediator of p53's biological effects (Raver-Shapira et al., 2007). Recently a negative regulatory loop involving *miR-221-222* and estrogen receptor α (ERα) was reported in breast cancer. High levels of *miR-221-222* were observed in ERα-negative ($ER\alpha^-$) cells and highly aggressive breast tumors. Upon confirmation that ERα is a direct target of *miR-221-222*, ERα was found to negatively regulate expression of *miR-221* and *miR-222* by promoter binding. This feedback loop seems to be a regulatory mechanism involved in the development of ERα-negative breast cancers (Di Leva et al., 2010). Through direct binding to promoter regions, c-myc can also modulate transcription of miRNA genes including over-expression of the *miR-17-92* cluster or repression of a subset of miRNAs such as *miR-15a*, *miR-29*, *miR-34* and the *let-7* family (Chang et al., 2008; O'Donnell et al., 2005). Interestingly, c-myc is in turn targeted and regulated by *let-7*, suggesting a regulatory double-feedback loop in human cancer (Sampson et al., 2007). Finally, activation of the transcriptional factor HIF (hypoxia-inducible factor) may cause over-expression of *miR-210* in human cancer (Giannakakis et al., 2008).

Alternatively, miRNA expression in cancer can also be affected by epigenetic changes, *e.g.* methylation of CpG islands in promoter regions. *miR-127* was the first example of a miRNA silenced by promoter hypermethylation in bladder cancer (Saito et al., 2006). In the following years, the important role of the DNA methylation in regulating miRNA expression has been described in various tumor types. Under-expression of *miR-34b/c* in colorectal cancer is associated with CpG islands methylation (Toyota et al., 2008) while methylation-mediated silencing of *miR-124* has been reported in cervical cancer (Wilting et al., 2010). Histone modifications have also been reported as possible regulatory mechanisms of miRNA expression in cancer cells (Nasser et al., 2008). Interestingly, miRNAs themselves can be regulators of epigenetics changes. Recent studies have reported that specific miRNAs can post-transcriptionally regulate components of DNA methylation machinery (e.g. DNA methyltranferases –DNMTs-)

affecting methylation patterns observed in cancer. The first evidence was provided in two studies showing that members of the *miR-29* family, including *miR-29a/b/c*, could directly target DNMT3A and DNMT3B affecting global DNA methylation in lung cancer and acute myeloid leukemia (AML) (Fabbri et al., 2007; Garzon et al., 2009). Further, *miR-148* was shown to target DNMT3B in HeLa cells by interacting with a binding site in the coding region, instead of the 3'UTR. More recently, DNMT1 was identified as a direct target of both *miR-148* and *miR-152* in human cholangiocarcinoma cells (Braconi et al., 2010). Nucleotide polymorphisms may also affect miRNA expression. Single nucleotide polymorphism (SNP) has been described in the *pre-miR-16* causing decreased miRNA processing in CLL (Calin et al., 2005). A guanine/cytosine polymorphism in the *pre-miR-146* was found to be associated with reduced mature miRNA levels and higher risk of papillary thyroid cancer (Jazdzewski et al., 2008).

The involvement of viruses in cancer development has also been proposed. As previously mentioned, viruses can encode miRNAs using the miRNA machinery from the host cells. These miRNAs might be expressed and abundantly accumulated in infected cells leading to an alteration of endogeneous miRNA processing, export or RISC function (Andersson et al., 2005). Viruses might also trigger changes in the host miRNA expression profile at transcriptional level (Yeung et al., 2005) or, alternatively, dsRNA-binding proteins produced by viruses might interfere with the processing of endogenous miRNAs as it has been observed in plants (Chapman et al., 2004). Although, not directly responsible of deregulation of endogenous miRNAs, it is worth to mention that virally-encoded miRNAs can directly act as oncogenes and stimulate cancer development and/or progression by targeting host transcripts with tumor suppressive function (Kim do et al., 2007; Pfeffer and Voinnet, 2006).

In addition, alteration of miRNA processing enzymes involved in miRNA biogenesis can influence miRNA expression and function in cancer. Over-expression of Drosha was observed in advanced cervical squamous cell carcinoma and was associated with changes in global miRNA expression (Muralidhar et al., 2007). Further, decreased TRBP2 levels, due to a frame-shift mutation, in colorectal cancer destabilizes the Dicer protein causing disruption of miRNA biogenesis (Melo et al., 2009). Recently, Martello *et al.* showed that *miR-103/-107* represses expression of Dicer in breast cancer by direct interaction to its 3'UTR. Lower levels of Dicer protein stimulated migration and metastasis through inactivation of *pre-miR-200* processing (Martello et al., 2010). Inactivating mutations in the exportin 5 gene were shown to interfere with pre-miRNA processing and lead to impaired mature miRNA production in cancer cells (Melo et al., 2010). Moreover, loss of Ago2 was associated with reduced expression and activity of mature miRNAs in human cells (Diederichs and Haber, 2007).

1.3.1.2 Oncogenic miRNAs

The first described miRNA with oncogenic properties was *miR-155* (Metzler et al., 2004), which derives from the primary transcript known as *BIC* (B-cell integration cluster). Over-expression of *miR-155* was reported in a wide range of hematological malignancies (Garzon et al., 2008; Kluiver et al., 2005) as well as in solid tumors such as lung, pancreatic and breast cancer (Greither et al., 2010; Volinia et al., 2006). However, little is known about regulatory mechanisms that drive *miR-155/BIC* over-expression. In line with the oncogenic potential of this miRNA, over-expression of *miR-155* in transgenic mice leads to development of acute lymphoblastic leukemia, through down-regulation of SHIP (Src- homology 2 domain-containing inositol-6-phosphatase) and c/EBPbeta (CCAAT enhancer-binding protein beta) (Costinean et al., 2009).

Concordant with the hypothesis that miRNAs are sitting in genomic fragile sites, the *miR-17-92* cluster (including *miR-17, miR 18a, miR 19a, miR-19b-1, miR-20a* and *miR-92-1*), is an example of miRNA polycistron with oncogenic functions located on human chromosome 13q31, a region frequently amplified in lymphomas (Ota et al., 2004). Members of the *miR17-92* cluster have been found to be highly expressed in many solid tumors and hematological malignancies (Mendell, 2008). Further, the *miR-17-92* cluster was shown to promote proliferation and angiogenesis and to accelerate, in cooperation with *c-myc*, tumor development in mouse models (Dews et al., 2006; He et al., 2005). Different studies have reported that the *miR-17-92* cluster affects cell cycle and proliferation regulating the E2F transcription factors family (O'Donnell et al., 2005). Additionally, it was shown that members of this cluster can modulate the expression of known tumor suppressors such as PTEN (phosphatase and tensin homologue), p21 and the apoptotic protein Bcl2-ineracting mediator of cell death (BIM) (Mendell, 2008). Interestingly, down-regulation of *miR-17-5p* was observed in breast cancer cell lines. Ectopic expression of this miRNA caused decreased cell proliferation in breast cancer cells by translational repression of *AIB1* (amplified in breast cancer gene 1) (Hossain et al., 2006). In addition, deletion of the *miR-17-92* chromosomal locus has been reported in breast cancer, ovarian cancer and melanoma (Hossain et al., 2006; Mendell, 2008). Taken together, these observations support the proposed hypothesis that the same miRNA might have a dual role, as oncogene or tumor suppressor, depending on the cellular context where it is expressed.

1.3.1.3 Tumor suppressor miRNAs

A clear example of a miRNA that acts as a tumor suppressor gene is given by the aforementioned *miR-15* and *-16*. They are transcribed as a cluster and their reduced expression has been reported not only in CLL but also in diffuse large B-cell

lymphoma (DLBCL), prostate cancer and pancreatic cancer (Bonci et al., 2008a; Calin et al., 2002; Eis et al., 2005). The suppressor function of the *miR-15a/16-1* cluster has been demonstrated in a number of studies showing that ectopic expression of these two miRNAs can dramatically reduce tumorigenicity in leukemic mouse models (Calin et al., 2008) and affect survival, proliferation and invasion of prostate cancer cells targeting *BCL2* (B-cell lymphoma 2), *CCND1* (cyclin D1) and *WNT3A* (wingless-type MMTV integration site family member 3A) (Bonci et al., 2008a).

The *let-7* family is also down-regulated in different tumor types (Iorio et al., 2005; Sempere et al., 2007; Takamizawa et al., 2004) and miRNA members of this family are located in fragile genomic sites associated with lung, breast and cervical cancer (Calin et al., 2004b). Moreover, functional studies support the tumor suppressor role of the *let-7* family in cancer development: high levels of *let-7* family members can inhibit the activity of well known oncogenes such as the Ras family, HMGA2 (high mobility group AT-hook 2) or c-myc and induce apoptosis and cell cycle arrest (Johnson et al., 2005; Lee and Dutta, 2007; Sampson et al., 2007).

Since these early findings, other miRNAs with a tumor suppressive function have been discovered. To mention a few, members of the *miR-29* family have been reported as deleted in cases of myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML). In CLL patients, mutations of the *miR-29* precursor, affecting its processing, have also been reported (Calin et al., 2005). Moreover, *miR-221* and *miR-222* were found to act as tumor suppressors in erythroblastic leukemia inhibiting cell growth through repression of the oncogene *KIT* (Felli et al., 2005). However, similar to the dual-function previously described for *miR-17-5p*, these two miRNAs can promote cell proliferation and inhibit apoptosis in various solid tumors suppressing the function of different tumor suppressors such as PTEN, p27, p57 and tissue inhibitor of metallo-proteinase 3 (TIMP3) (Fornari et al., 2008; Garofalo et al., 2009; le Sage et al., 2007).

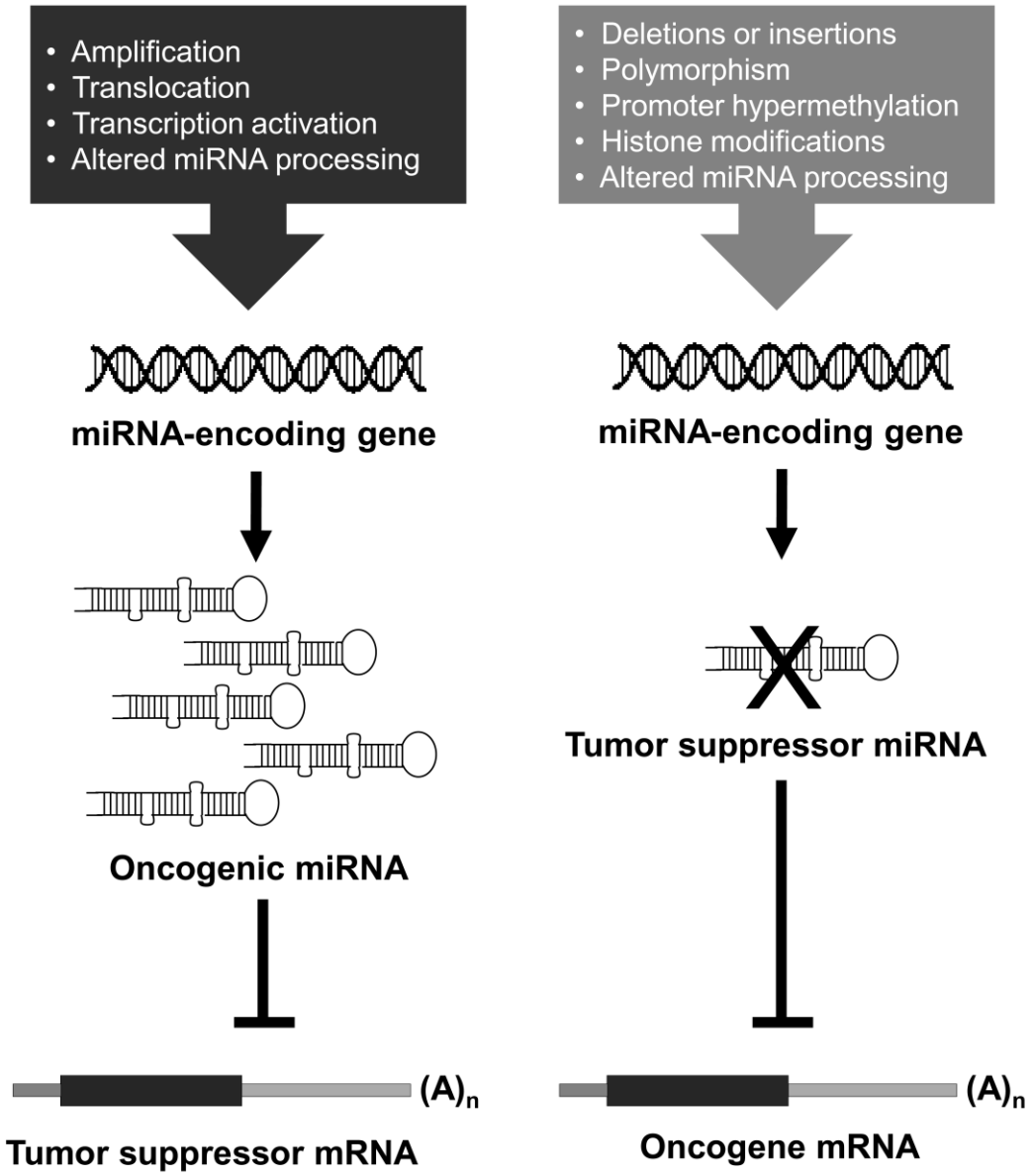


Figure 2. miRNAs as oncogenes or tumor suppressors. This model shows that miRNAs which down-regulates tumor suppressor genes are considered as oncogenes and are usually over-expressed in cancer (left). On the other hand, miRNAs which down-regulates expression of oncogenes are considered as tumor suppressors and are likely to be lost or inactivated in cancer (right). Possible regulatory mechanisms affecting miRNA expression are indicated at the top.

1.3.2 Diagnostic and prognostic value of miRNAs

In addition to the potential role as oncogenes or tumor suppressors in tumor development, a number of recent studies have proposed the use of miRNA expression profiles as molecular tools in tumor diagnosis and prognosis. Volinia *et al.* (Volinia et al., 2006) performed a large genome-wide miRNA profiling study on 540 samples including breast, lung, stomach, prostate, colon and pancreatic tumors. The results showed specific miRNA signatures associated with these six solid tumor types. On the other hand, some miRNAs were commonly deregulated between different cancers suggesting that they might have an essential role in carcinogenesis. Another large miRNA profiling study was performed by Lu and colleagues (Lu et al., 2005) using a bead-based hybridization technology. They evaluated the expression of 217 miRNAs in a group of 334 samples including multiple tumor types (mainly hematological malignancies) and non-neoplastic tissue as normal reference. Their findings revealed a general miRNA deregulation in tumor samples as compared to normal tissue and the presence of specific miRNA signature that could classify the tumor samples according to their tissue of origin. Moreover, deregulated miRNAs could clearly distinguish molecular subtypes or classify poorly differentiated tumors.

Several studies have also shown the prognostic potential of miRNA expression in solid tumors and hematological malignancies. To mention a few, high expression of *miR-155* and low levels of *let-7a* was associated with short survival in lung cancer patients (Yanaihara et al., 2006). Increased expression of *miR-21* was identified as an independent factor associated with short overall survival in colon cancer (Schetter et al., 2008) and a set of five up-regulated miRNAs was associated with poor outcome in AML patients (Garzon et al., 2008). In a recent study, Segura *et al.* identified a signature of 18 up-regulated miRNAs which was associated with longer survival in metastatic melanoma patients. In addition they constructed a classifier of 6 miRNAs that could predict post-recurrance survival in the same cohort (Segura et al., 2010). Further, low expression of *miR-195* or increased expression of *miR-483-5p* in adrenocortical carcinoma (ACC) cases was associated with poorer disease-specific survival (Soon et al., 2009).

Overall, miRNA profiling studies have shown the presence of miRNA signatures to distinguish tumors from normal tissues or to sub-classify tumors based on specific clinicopathological features. These differences in terms of miRNA expression appear to be tumor and tissue specific and enable to accurately classify and diagnose cancer patients and in some cases to predict their outcome.

In addition, miRNAs have recently been shown to be released in the peripheral circulation by cancer cells and can stably be present in the blood either contained within exosomes (Hunter et al., 2008; Taylor and Gercel-Taylor, 2008) or bound to Ago2 complexes (Arroyo et al., 2011). Interestingly, a number of studies identified miRNA signatures in serum and plasma that could distinguish patients with cancer from healthy controls in variety of tumor types including melanoma, DLBCL, ovarian and lung cancer (Kanemaru et al., 2011; Lawrie et al., 2008; Rabinowits et al., 2009; Taylor and Gercel-Taylor, 2008). To give an example, in a cohort of 94 melanoma patients, Kanemaru *et al.* observed that the plasma levels of *miR-221* were significantly higher as compared to healthy controls. In addition, after surgical removal of the primary tumor, the levels of *miR-221* in the plasma were notably decreased. Taken together, these data support the value of circulating *miR-221* as a potential diagnostic biomarker in melanoma as well as a valuable marker to follow up melanoma patients after surgery (Kanemaru et al., 2011). Despite the promising findings in different tumor types, the prognostic and diagnostic potential, the functional implications and the optimal detection methods for circulating miRNAs are yet to be elucidated.

1.4 MiRNAs as therapeutic targets

The key role of miRNAs in tumor initiation and progression makes these small molecules really attractive candidates for development of therapeutic drugs. The potential advantages of using miRNA-based therapies in cancer treatment are today being widely explored. The strategies adopted to target miRNA expression in cancer involve the use of oligonucleotide or virus-based constructs to either inhibit the expression of oncogenic miRNAs or reintroduce tumor-suppressor miRNAs lost in cancer (Figure 3).

1.4.1 Inhibition of oncogenic miRNA expression

1.4.1.1 Antisense oligonucleotides (or antagomirs or anti-miRNAs)

Antisense oligonucleotides act as inhibitors of miRNAs, binding to the mature miRNA with perfect complementarity. This interaction leads to a duplex formation, which sequesters the miRNAs from its mRNA targets and in some cases can induce degradation of the targeted miRNA. Chemical modifications of the oligonucleotides structure have been adopted in order to increase their stability, binding affinity and specificity.

The first type of modification consists of the introduction of a 2'-O-methyl (2'-OMe) group which improves the binding affinity to RNA and partly contributes to nuclease resistance. In alternative, 2'-O-methoxyethyl (2'-OME)-modified oligonucleotides show

a higher affinity and specificity compared to their 2'-OMe analogs. The efficacy of these modifications was first shown by Krutzfeldt and collaborators who developed a 2'-OMe-modifed cholesterol-conjugated antagomir against *miR-122*, a highly abundant and liver-specific miRNA. Intravenous administration of this modified antagomir in mice resulted in a specific, efficient and long-lasting (about 3 weeks from antagomir injection) silencing of *miR-122*. In addition, using *miR-16* as target, given its abundance in all tissues, they demonstrated that antagomirs *in vivo* can reach a wide bioavailability and can efficiently silence miRNAs in most tissues (Krutzfeldt et al., 2005).

Another common modification is based on the incorporation of locked nucleic acid (LNA) monomers into the chemical structure of antagomirs. LNAs are conformationally 'locked' nucleotides with a methylene bridge connecting the 2'-Oxygen and 4'-Carbon in the ribose ring. This conformation provides a significantly increased nuclease resistance, stabilizes the duplex structure and improves mismatch discrimination (Braasch et al., 2003). LNA-modified oligonucleotides have been shown to mediate specific inhibition of miRNA function both *in vitro* (Orom et al., 2006) and in mice (Elmen et al., 2008b). Recently, Elmen *et al*. (Elmen et al., 2008a) has shown for the first time that LNA-antagomirs can effectively silence miRNAs also in non-human primates. This pioneering study in primates confirmed that LNA-modified antisense oligonucleotides are specific, stable and non-toxic when administrated intravenously, supporting the great potential of these compounds as therapeutics tools.

1.4.1.2 miRNA sponges

miRNA sponges (or decoy) are transcripts that contain multiple binding sites (in tandem) to a miRNA of interest and are transcribed from mammalian transcription vectors (plasmids or vectors based on lentiviruses or retroviruses). When introduced to the cell, miRNA sponges can saturate an endogenous miRNA and prevent the regulation of its natural targets. By introducing a bulge in the decoy transcripts at the position normally cleaved by Ago2, it is possible to increase the affinity of these miRNA sponges and stabilize the association with the miRISC (Ebert et al., 2007). Further, a single miRNA sponge can be used to silence an entire family of miRNAs that share the same seed sequence (Ebert et al., 2007). The silencing effect obtained by these sponges is comparable to those obtained by 2'-OMe- or LNA-modified oligonucleotides. However, unlike oligonucleotides-based miRNA silencing, miRNA sponges can stably inhibit miRNA expression without the need of multiple administrations. In addition, miRNA sponges can stably integrate into chromosomes or can be designed in order to become drug inducible or be under control of specific promoters whose expression is cell or tissue specific.

1.4.1.3 miR-mask antisense oligonucleotides

The miRNA masking technology is a gene-specific strategy developed by Xiao and colleagues (Xiao et al., 2007). In contrast to miRNA sponges, a miR-mask is a single-stranded 2'-O-methyl modified antisense oligonucleotide which does not directly interact with a specific miRNA but, instead, is fully complementary to the binding sites of that miRNA in the 3'UTR of the target mRNA. Thus, these modified oligonucleotides compete with an endogenous miRNA and mask the access to the binding sites of the target mRNA. Although off-target effects are reduced with this approach, the gene-specificity of miR-masks can be a limitation for therapeutic purposes since miRNAs exert their function through repression of multiple targets to contribute to tumorigenicity.

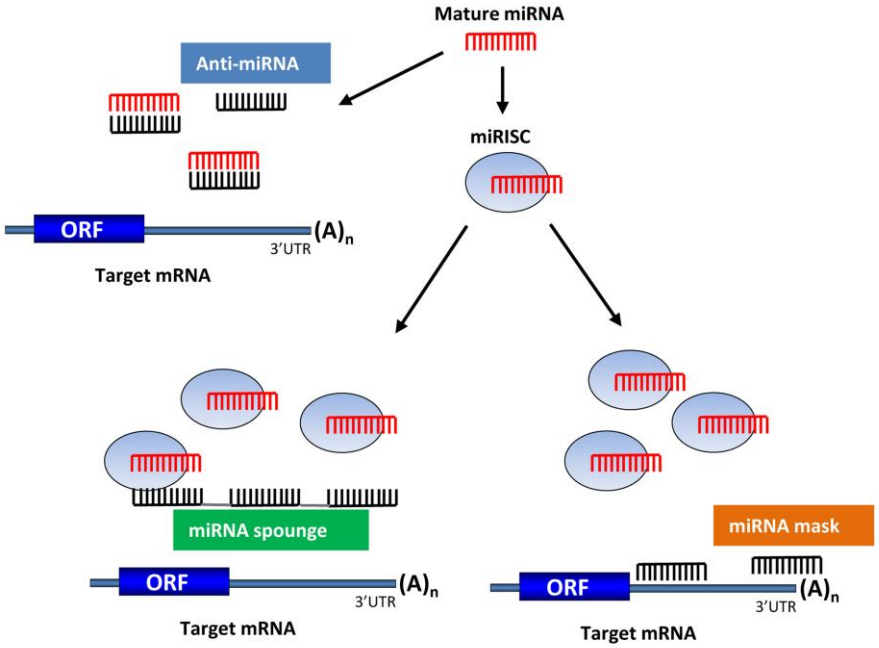
1.4.2 Restoration of expression for tumor-suppressor miRNAs

1.4.2.1 miRNA mimics and adenovirus-associated vectors (AVV)

The use of synthetic oligonucleotides mimicking endogenous mature miRNA is a valuable approach to restore the expression of tumor suppressor miRNAs which are lost or down-regulated in cancer. These miRNA mimics are short, double stranded and 2'-O-methyl chemically modified oligonucleotides. Some of them present a longer sequence such as miRNA precursor (*e.g.* pre-miRNA developed by Ambion). Several studies have shown how miRNA mimics can repress proliferation and promote cell death in cancer cell lines or effectively decrease tumorigenicity in mouse models (Bonci et al., 2008b; Garzon et al., 2009; Xiong et al., 2010). However there are presently no *in vivo* studies demonstrating whether miRNA mimics could be efficiently delivered by intravenous injection to be suitable as therapeutic tools.

Adenovirus-associated vectors (AVV) represent an alternative strategy to re-establish the expression of tumor suppressor miRNAs in cancer cells. AVVs show a highly efficient gene transduction to target cells, do not integrate into the genome and can be efficiently eliminated with a minimal effect of toxicity as shown in clinical trials (Michelfelder and Trepel, 2009). Further, a recent study showed that systemic administration of *miR-26a* in a murine liver cancer model using an AVV resulted in apoptosis induction and cell growth inhibition without any sign of toxicity. This is the first report showing that restoration of tumor suppressor miRNA expression could repress tumor progression *in vivo*. Although this approach might be valuable for treatment of liver cancer, the efficacy of these virus-based vectors in other tumor types and other organs is still unknown (Kota et al., 2009).

A



B

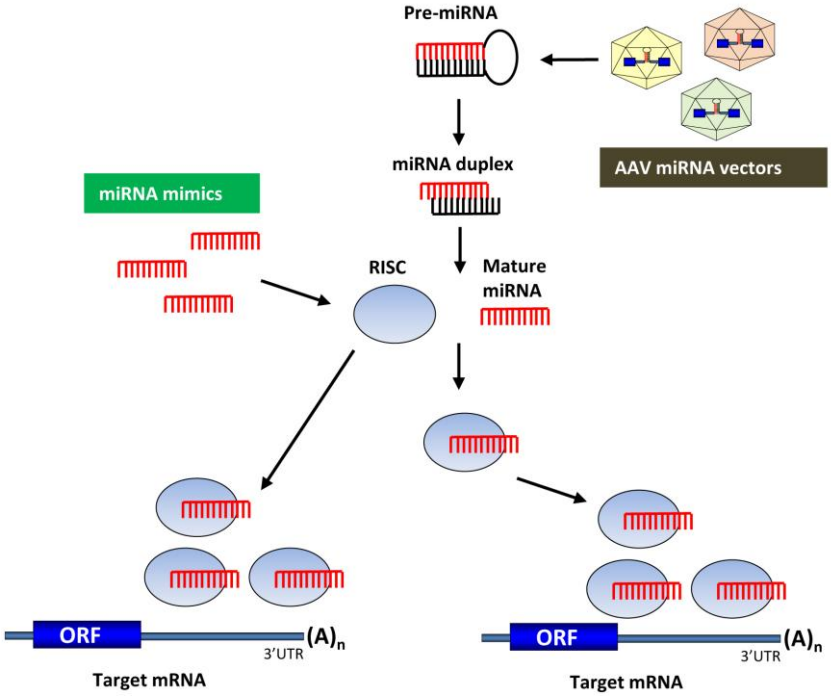


Figure 3. miRNA-based approaches to modulate expression of oncogenic miRNAs (A) or tumor suppressor miRNAs (B). miRISC, miRNA-induced silencing complex; ORF, open reading frame; UTR, untranslated region; AAV, adeno-associated virus.

1.5 Strategies for miRNA analysis

A number of molecular technologies are today available for discovery, identification, detection and characterization of miRNA species.

1.5.1 Biological approaches to detect miRNAs

1.5.1.1 Hybridization-based techniques

1.5.1.1.1 Northern blot analysis

Northern blot is a standard method for detection of RNA and it can be a valuable choice for miRNA identification after some modifications. Given the small size, in order to obtain a better size-based separation, polyacrylamide gels are a preferable choice rather than agarose gels. Further, radio-labeled (*e.g.* ^{32}P) locked nucleic acid (LNA) probes are often used to increase the sensitivity (Varallyay et al., 2008). Since this technique involves a size-based separation, it has the advantage of detecting pri-miRNA, pre-miRNA and the mature form in a single hybridization. However, this method is time consuming and is not very sensitive. It requires high amount of RNA (5-10µg) limiting its use for analysis of miRNAs in clinical material. Moreover, using this method, it can be difficult to clearly distinguish between miRNAs belonging to the same family which differ in only one nucleotide (*e.g. let-7* family). In addition it is limited to detection of known miRNAs.

1.5.1.1.2 *In situ* hybridization

This is an alternative method used for miRNA detection in cells or tissue sections. The procedure is similar to conventional *in situ* hybridization used for mRNA detection with differences in the probe preparation. In both cases digoxygenin (DIG) is used to label the probe but for mRNA, DIG is incorporated into the probe (internal labeling) while for miRNA, due to the small size, DIG is added to the 3' end of the probe using deoxynucleotidyl transferase. In order to improve the specificity, LNAs are also incorporated into the probe. This method allows both detection and cellular localization of the miRNA of interest. However its sensitivity is relatively low and it is limited to detection of known miRNAs.

1.5.1.1.3 Microarray analysis

Compared to Northern blot and *in situ* hybridization techniques, miRNA-microarray is a high-throughput technology which is widely used in miRNA expression profiling studies. Importantly, microarrays can contain probes with different design to detect both precursor and mature forms of the miRNA on the same chip (Liu et al., 2004). Although, miRNA-microarray is a simple, efficient and relatively low-cost method to obtain miRNA expression profiles, only known miRNAs can be identified.

1.5.1.2 Quantitative Real-Time PCR (qRT-PCR)

This method, normally used for detection of DNA or complementary DNA (cDNA), has been adapted for detection of mature miRNA molecules. Since they are short and do not contain a poly(A) tail at the 3' end, alternative strategies have been developed for the cDNA synthesis such as the use of stem-loop primers that hybridize to the 3' end of the miRNA. Alternative but less sensitive PCR methods based on poly(A) tailing for cDNA synthesis and SYBR-Green detection have also been developed (Chen et al., 2011). However, qRT-PCR based on stem-loop primers is the most commonly used and allows a fast, easy and sensitive detection of miRNAs using a small amount of RNA (about 100ng). Furthermore, the stem-loop structure gives the advantage to discriminate between miRNAs that differ by only one nucleotide. qRT-PCR is also considered as the *'gold-standard'* method for validation of microarray results. At present, stem-loop qRT-PCR assays for miRNA are also available as low-density arrays giving the possibility to analyze the expression of 384 miRNAs on a single plate. However, qRT-PCR is limited to the analysis of known miRNAs.

1.5.1.3 High throughput sequencing

Recently, different high throughput sequencing platforms (Illumina, 454 from Roche and SOLiD from Applied Biosystem) became available to researchers to sequence and profile small RNA populations. Different from the previously mentioned techniques, these sequencing methods are useful tools for the discovery of novel miRNAs. In addition, this technology allows to quantify and detect miRNA sequence variations (isomirs) in mature miRNAs, including RNA editing (Blow et al., 2006) and 5'/3'-end variations (Morin et al., 2008). All these sequencing platforms can provide enormous amounts of information although differences can apply for each technology in terms of number of small RNAs that can be profiled, the sequencing error rate and the effective cost. Undoubtedly, in these past few years, the deep sequencing technology has accelerated the biomedical research enabling a more comprehensive analysis of the genomes. However, at present, the elevated costs and the requirement of high bio-informatics expertise for data management are still limitations for these high-throughput technologies to become widespread and routinely used.

1.5.2 Tools for identification of miRNA targets

While there are many technologies available for detection and identification of miRNA, our knowledge on their function is still limited. In order to understand the regulatory role of miRNAs, computational and biological strategies for target prediction and validation have been developed. However the interaction between miRNA and mRNA-target is a complex process making it more difficult to understand the mechanism of action of miRNAs. The major challenge to predict miRNA targets is due to the fact that

in animals (differently from plants) the miRNA have generally a limited complementarity to their targets (Ambros, 2004). The 'seed-region' at the 5' end of the miRNA, plays a major role in the specific interaction between miRNA and their targets and different kind of interactions can occur: i) Perfect base pairing of the seed region with an additional extensive interaction in the 3' end of the miRNA; ii) Perfect complementary interaction of the seed region but poor pairing in the 3' end; iii) Presence of a mismatch in the seed region with compensation of a more extensive complementary in the 3' end of the miRNA (3' compensatory) (Bartel, 2009; Maziere and Enright, 2007).

1.5.2.1 Computational target-prediction methods

In the attempt to identify miRNA targets a number of web-based programs have been developed. All these *in silico* methods apply different criteria for miRNA targets prediction such as perfect or nearly-perfect base pairing at the seed region (*e.g.* miRanda, TargetScan), evolutionary conservation of the seed sequence (*e.g.* TargetScan, PicTar), presence of multiple binding sites in the same target (e.g. TargetScan, miRanda), absence of secondary structure at the binding site of the target and favorable miRNA-target duplex thermodynamics (*e.g.* RNAhybrid, PicTar). Today it is possible to access individual softwares, and there are also online servers that integrate different prediction algorithms together (*e.g.* GOmiR, miRecords, miRGator). However, this integration often performs poorly as compared to the prediction by one single algorithm due to a significantly reduced sensitivity (Alexiou et al., 2009). Although, the identification of predicted miRNA target can be easy and relatively quick using the *in silico* approaches, it requires validation by biochemical techniques. The most commonly used programs for target prediction are summarized in Table 1.

1.5.2.2 Experimental methods to identify and/or validate miRNA-target interaction

The luciferase reporter assay is the approach most commonly used for identification of miRNA targets. A complete or partial 3'UTR sequence or an oligonucleotide containing multiple binding sites (in tandem) for the miRNA under investigation are cloned downstream of the luciferase gene contained in the reporter plasmid. The recombinant plasmid is transiently transfected in the host cells with or without over-expressing the miRNA of interest. When the candidate gene is an authentic target, the luciferase activity is decreased in the miRNA over-expressing cells as compared to the control cells.

Another valuable technique to identify targets and uncover the biological function of canditate miRNAs is based on cross-linking combined with co-immunoprecipitation (CLIP). In principle Ago2 complexes and bound RNAs are cross-linked by ultraviolet light (UV) and the Ago2-RNA complexes are purified by immunoprecipitation. The

immunoprecipitated complexes contain both miRNA and potential mRNA target. The isolated RNAs can be analyzed using high throughput sequencing, microarray or qRT-PCR.

Based on the principle that miRNAs can induce mRNA degradation or translational repression, microarray and proteomics have been adopted to identify miRNA targets. These high-throughput methods allow detection of global changes in mRNA or protein expression respectively, in presence or absence of the miRNA of interest. However, microarray techniques will provide significant information for miRNA target when gene expression is regulated by mRNA degradation but will miss those targets which are regulated by translational repression. Instead these targets can be identified using a proteomics approach (Selbach et al., 2008). The method adopted by Selbach *et al*. is based on the stable isotope labeling by amino acids added in cell culture for short time (pulsed-SILAC) and relies on the incorporation of labeled amino acids into newly synthesized proteins. Two different isotopes, 'heavy' and 'medium-heavy', are added to the culture medium of the control cells and cells over-expressing the miRNA of interest. During labeling, all newly synthesized proteins will be 'heavy' or 'medium-heavy', while the pre-existing proteins present before labeling will remain in the light form. The miRNA targets can be identified by the ratio of heavy and medium-heavy isotope signal intensity. The signal intensity for the medium-heavy isotope will decrease whenever the mRNA is a target of the miRNA investigated, otherwise the two types of isotopes will produce similar signal intensity. However, it should be noted that both microarray and proteomics-based methods provide indirect validation of miRNA target and the results still have to be verified using alternative biochemical approaches.

Table 1. Algorithms for miRNA target prediction

Program	Organisms	Prediction features	Availablity on the Web	Reference
DIANAmicroT	Human, mouse, rat, drosophila, nematode, arabidopsis	Thermodynamics, conservation	http://diana.cslab.ece.ntua.gr/microT/	Maragkakis et al., 2009
EIMMo	Human, drosophila, zebrafish, nematode, mouse	Conservation	http://www.mirz.unibas.ch/ElMMo2/	Gaidatzis et al., 2007
miRanda	Human, drosophila, zebrafish	Complementarity, conservation	http://www.microrna.org	Enright et al., 2003
miTarget2	man, mouse, rat, dog, chicken	SVM classifier	http://mirdb.org/miRDB/index.html	Wang and El Naqa, 2008
PicTar	Vertebrates	Seed region complementarity, conservation, thermodynamics	http://pictar.mdc-berlin.de/	Krek et al., 2005
Rna22	Any	Pattern-based identification, thermodynamics	http://cbcsrv.watson.ibm.com/rna22.html	Miranda et al., 2006
RNAhybrid	Mammals	Thermodynamics	http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/	Rehmsmeier et al., 2004
TargetScan 6.0	Mammals, worm, drosophila	Seed region complementarity, conservation	http://www.targetscan.org	Lewis et al., 2005; Grimson et al., 2007 Friedman et al., 2009
PITA	Human, mouse, fly, worm	Complementarity, thermodynamics	http://genie.weizmann.ac.il/pubs/mir07/ mir07_prediction.html	Kertesz et al., 2007

SVM, support vector machine

2 AIMS OF THE STUDY

The overall objectives of this thesis were to determine deregulation of miRNAs and miRNA machinery components in human cancer and to assess their role in tumor development and progression. Moreover, we aimed to evaluate the prognostic and diagnostic impact of their deregulation in human malignancies and possible applications in clinical practice. More specifically, we aimed to:

Paper I. Explore the impact of microRNA deregulation in malignant melanoma by analyzing miRNA expression data in relation to clinical, molecular and histopathological characteristics.

Paper II. Identify miRNA classifiers to distinguish adrenocortical carcinomas (ACC) from adenomas, evaluate the potential of deregulated miRNAs as molecular predictors of survival among ACC cases and elucidate the role of specific miRNAs in the pathogenesis of ACC.

Paper III. Evaluate the expression levels of core components of the miRNA machinery, to define their value as diagnostic and/or prognostic biomarkers in adrenocortical tumors and explore their role in development or progression of ACC.

Paper IV. Investigate the impact of miRNA and miRNA machinery deregulation in diffuse large B-cell lymphoma (DLBCL) and unravel their functional role in DLBCL pathogenesis.

3 MATERIALS AND METHODS

3.1 Patients, clinical material and cell lines

In this thesis, clinical tissue samples and established cell lines were used to study human tumors including metastatic melanoma, adrenocortical tumors and diffuse large B-cell lymphoma (DLBCL).

Tissue material for metastatic melanoma was collected at the Karolinska University Hospital in Sweden while melanoma cell lines were kindly provided by Dr. Monica Rodolfo (INT, Milan, Italy) (**Paper I**). Adrenocortical tumor samples and normal adrenocortical cortices were obtained from the Karolinska University Hospital in Sweden (**Papers II** and **III**). DLBCL cases were received from Uppsala University Hospital in Sweden and the anonymous reactive lymph nodes were obtained from Karolinska University Hospital and Uppsala University Hospital (**Paper IV**).

Twenty-one non-commercially available melanoma cell lines were included in **Paper I**. The cells were derived from primary melanomas, lymph node metastases or cutaneous metastases and were propagated in culture for 18 passages in average. The NCI-H295R adrenocortical cancer cell line, used in **Paper II** and **III**, was purchased from the American Type Culture Collection (ATCC; LGC Standards, Middlesex, UK). The four established *de novo* DLBCL cell lines (OCI-Ly-1, OCI-Ly-3, OCI-Ly-7 and OCI-Ly-10) in **Paper IV** were kindly provided by Dr Mark Minden (University Health Network, Toronto, Canada). OCI-Ly-1 and OCI-Ly-7 are GCB-subtype while OCI-Ly-3 and OCI-Ly-10 are ABC-subtype.

3.1.1 Cutaneous metastatic melanoma (Paper I)

Cutaneous metastatic melanoma is a highly aggressive tumor, which originates from melanocytes and is responsible for the majority of skin tumor-related deaths worldwide. Despite the improvement in our understanding of biological processes of malignant melanoma tumorigenesis, the prognosis of patients with advanced disease is still poor (Garbe and Eigentler, 2007) and early diagnosis, based on Breslow's tumor thickness and sentinel lymph node status, together with early surgical removal is still the best treatment to reduce mortality. Activating *BRAF* mutations (V600E), which affect the mitogen-activated protein kinase (MAPK) pathway, are present in 60-70% of malignant melanomas (Davies et al., 2002). *NRAS* mutations are also detected in 15-30% of melanoma tumors (Chin et al., 1999) and cause constitutive activation of the MAPK pathway independently of BRAF. Inhibitions of mutated BRAF or NRAS are promising and novel therapeutic tools for the treatment of malignant melanoma.

In this study, a total of 32 fresh frozen regional lymph node metastases and 21 melanoma cell lines were analyzed in terms of miRNA expression. Human melanocyte cultures (PromoCell, Heidelberg, Germany) from three different individuals were included as non-neoplastic reference.

3.1.2 Adrenocortical carcinoma (Papers II and III)

Adrenocortical tumors derive from the cortex of the adrenal gland and are often incidentally detected as biochemical and clinically asymptomatic adrenal masses. The majority of these tumors are adenomas, which can be hormonally inactive or over-produce *e.g.* aldosterone (aldosteronoma) or cortisol (Cushing), while only a small proportion is classified as carcinomas (ACC). While the annual incidence is low with 4-12 cases per million among adults (Grumbach et al., 2003), ACC is an aggressive tumor associated with poor prognosis (Bertherat et al., 2006). Further, in the absence of invasion and/or distant metastases, the distinction between ACC and adrenocortical adenomas can be challenging and relies on histopathological evaluation and Weiss score classification (Weiss et al., 1989). Moreover, prognostic and diagnostic biomarkers for early detection of ACC are still lacking in clinical practice.

A variety of studies have recently shown the potential of mRNA and miRNA profiling to correctly classify adenomas and carcinomas and to identify subgroups of ACC patients with different survival and outcome. To date over-expression of the insulin-like growth factor 2 gene (*IGF2*) was observed in more than 80% of the ACC cases. Expression levels of *DLG1* (discs large homolog 1) and *PINK1* (PTEN induced putative kinase 1) genes were associated with clinical outcome in ACC. Further, increased expression of *miR-483-3p/-5p* and under-expression of *miR-195* was reported in ACC cases. (de Fraipont et al., 2005; de Reynies et al., 2009; Giordano et al., 2009; Laurell et al., 2009; Ozata et al., 2011; Patterson et al., 2011; Soon et al., 2009; Tombol et al., 2009).

In our studies, 73 frozen primary sporadic tumors from 72 patients were included. In addition, 10 histopathologically verified normal adrenal cortical samples were used as non-neoplastic references. These samples were obtained from 10 patients undergoing nephrectomy for other reasons at the Karolinska University Hospital.

3.1.3 Diffuse large B-cell lymphoma (Paper IV)

Diffuse large B-cell lymphoma (DLBCL) is a highly aggressive lymphoma. It is the most common type of lymphoma accounting for approximately 40% of B-cell lymphoma cases among adults in the Western countries (Coiffier, 2001). The majority

of DLBCLs occur as *de novo* while a small proportion can progress from a previous indolent lymphoma such as follicular lymphoma. DLBCL is a heterogeneous group of lymphomas and is characterized by numerous chromosomal aberrations and genomic alterations. Among others, the translocation t(14;18)(q32;q21) which induces BCL2 over-expression is frequently found (Rantanen et al., 2001). Rearrangements involving the locus for *BCL6* in 3q27 or somatic mutations in its regulatory sequences have also been reported (Pasqualucci et al., 2001). Further, mutations in the *TP53* gene have been described in DLBCL and associated with poor prognosis (Leroy et al., 2002). In addition, based on mRNA or miRNA expression profiling, DLBCL can be genetically classified in three major subtypes: germinal center B cell-like (GCB), activated B cell-like (ABC) and type 3 of which the GCB subgroup shows a better prognosis (Alizadeh et al., 2000; Lawrie et al., 2007; Malumbres et al., 2009; Rosenwald et al., 2002).

In this study, 75 frozen tumor biopsies from primary DLBCL cases were analyzed. Paraffin embedded material for 22 specimens from the same series were also collected for immunohistochemical analysis. Among the tumor cases, 56 were *de novo* DLBCL while 19 were classified as transformed from follicular lymphoma. Ten anonymized samples of reactive lymph nodes were included in the study as references of non-neoplastic lymphatic tissue.

3.2 Methods

3.2.1 Microarray (Papers I, II and IV)

miRNA microarray is a high-throughput method for studying miRNA expression profiles in cultured cells or tissues. This is an efficient method, which offers the possibility to measure the expression of all known miRNAs simultaneously in a large cohort of samples at a relatively low cost.

In **Papers I**, **II** and **IV**, global miRNA expression profiling was performed using the Agilent human miRNA microarray platform (Agilent, Santa Clara, CA). This platform is a sensitive microarray-based assay, which requires very low amount of RNA input (100ng of total RNA) without the need of additional amplification or size fractionation steps. The labeling method is based on an enzymatic reaction (T4 RNA ligase) that attaches a single fluorophore-labeled nucleotide (pCp-Cy3) to the 3' end of each miRNA. This novel probe design allows a highly efficient detection of mature miRNAs. The probes are characterized by the addition of a guanosine (G) to their 5' end, which is complementary to the cytosine (C) added to the 3' end of each miRNA during the labeling. This G-C interaction allows to reach a melting temperature of about 55°C for almost all mature miRNAs. Different length of the probes is an additional

mean to normalize the melting temperature for all different miRNAs. Furthermore, a hairpin structure is present at the 5' end of the probes. This particular conformation destabilizes the hybridization to larger non-target RNAs and stabilizes the probe-target interaction. Features of the probe design are shown in Figure 4.

The experimental procedure included in brief: 120ng of total RNA were labeled with cyanine 3-pCp, hybridized onto arrays for 18-20h at 55°C, followed by washings according to the manufacturer's instructions. Slides were subsequently scanned using an Agilent microarray scanner G2565B and the images were processed with Feature Extraction Software v10.7.3.1 (Agilent). The miRNA intensity values were normalized and median centered using Cluster 3.0 software (de Hoon et al., 2004). Hierarchical clustering and generation of heat maps was performed with Cluster 3.0 and visualized through Treeview v1.60 (http://rana.lbl.gov/EisenSoftware.htm).

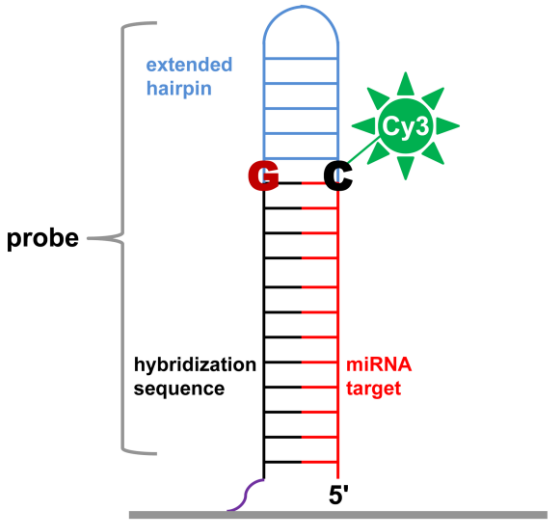


Figure 4. Schematic illustration of the probe design for the Agilent miRNA microarray platform [Modified from (Wang et al., 2007)].

3.2.2 Quantitative real-time PCR (qRT-PCR) (Papers I-IV)

Quantitative real-time PCR is a method to determine the amount of DNA or cDNA in a given sample through a PCR reaction. There are two types of qRT-PCR: one is a probe-based real-time PCR, which requires a set of specific primers and an additional fluorogenic probe (*e.g.* TaqMan PCR; Applied Biosystems, Foster City, CA). The other category includes intercalator-based PCR, also called SYBR Green, which involves the use of a dye, which binds to the newly synthesized double-stranded DNA during the PCR reaction and gives fluorescence. Although more expensive, the TaqMan method is

more accurate compared to SYBR Green. The TaqMan and SYBR Green methods are illustrated in Figure 5.

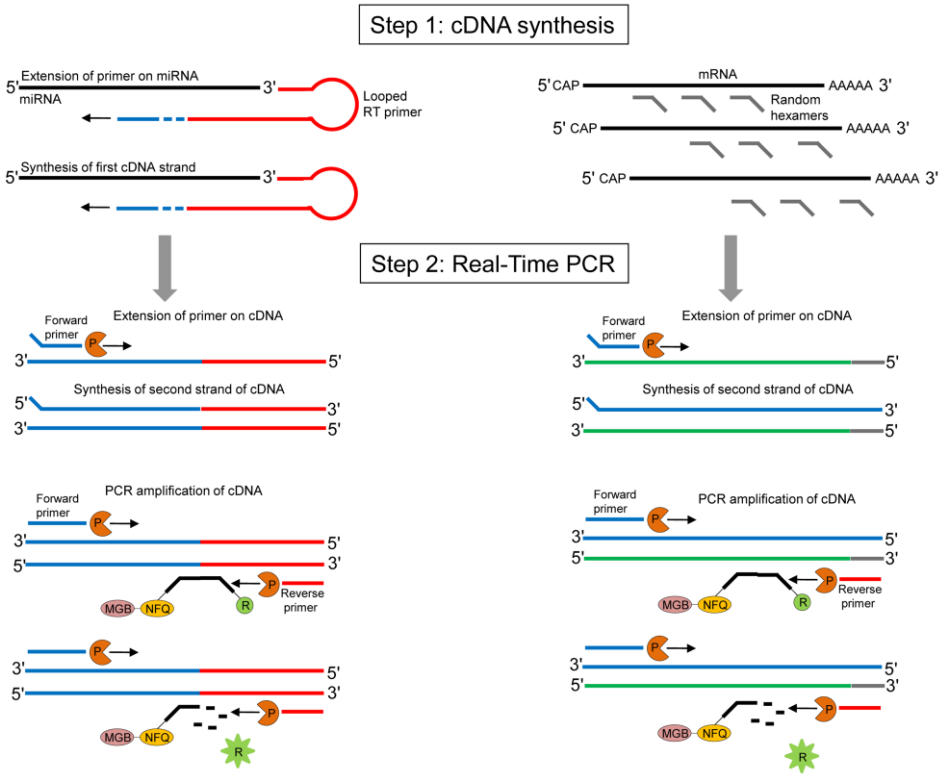
In **Papers I**, **II** and **IV** microarray results were validated by quantifying the expression levels of selected mature miRNAs by qRT-PCR. In addition, in **Papers III** and **IV**, quantitative real-time PCR was adopted to evaluate mRNA expression of specific genes of interest (*e.g. DROSHA*, *DICER*, *TRBP2*, *DGCR8*, *PACT*, *IGF2* and *H19*). Quantification of miRNAs using TaqMan assays follows a two-step RT-PCR procedure: Step 1) cDNA is synthesized from 25ng of total RNA using a stem-loop RT primer specific to the miRNA of interest and reagents contained in the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystem). The structure of the primer makes the assay more specific and provides a longer template for the following step. Step 2) During the PCR step, cDNA is amplified using predesigned TaqMan MicroRNA Assays (Applied Biosystems). The peculiarity of TaqMan assays consists in the presence of a TaqMan minor groove binder (MGB) probe which, during PCR, anneals specifically to the target between the forward and the reverse primer. The probe contains a reporter dye (FAM-dye) at the 5' end and a MGB linked to a non-fluorescent quencher (NFQ) at the 3' end. During the amplification step, the DNA polymerase cleaves the probes that are hybridized to the template causing the separation of the reporter from the quencher, which results in increased fluorescence. In our studies, each miRNA analyzed was normalized to the expression levels of *RNU6B* (RNA, U6 small nuclear 2). Analysis of mRNA expression follows the same principle; however higher amount of starting material is required (100ng of total RNA) and random hexamers are used for the cDNA synthesis (included in the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR adopts specific primers for each gene of interest. In our studies, normalization was done against *18S*.

In order to quantify the relative expression of primary and precursor forms of two miRNAs (*miR-155* and *miR-146a*), we adopted SYBR Green approach as previously described (Schmittgen et al., 2004). The expression levels of pre-miRNAs were calculated based on the following formula:

$$\text{Pre-miRNA} = 2^{-CT\,(\text{pri-miRNA + pre-miRNA})} - 2^{-CT\,(\text{pri-miRNA})}$$

Further, the processing efficiency was calculated as the ratio of mature to precursor for both *miR-155* and *miR-146a*. In all studies the relative expression levels for both miRNAs and mRNAs were determined using C_T method and reported as $2^{-\Delta CT}$.

A



B

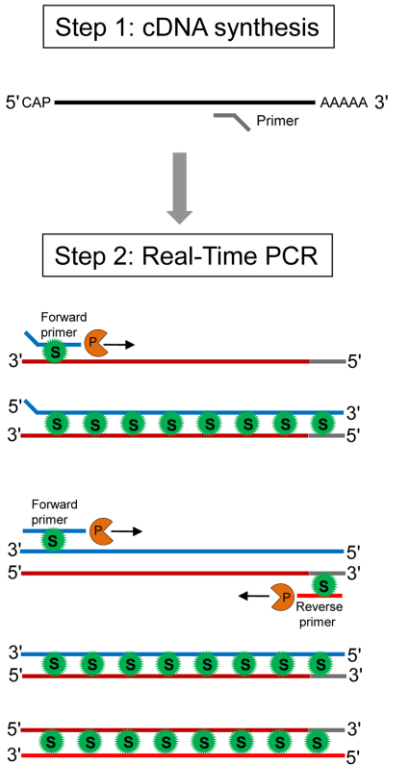


Figure 5. Chemistry of TaqMan and SYBR Green qRT-PCR methods. (A) Illustration of TaqMan two steps qRT-PCR method to quantify miRNAs (left) or mRNAs (right). P, DNA polymerase; NFQ, non-fluorescent quencher; MGB, minor groove binder; R, reporter. (B) Illustration of SYBR Green qRT-PCR. P, DNA polymerase; S, SYBR Green dye.

3.2.3 Western blot analysis (Papers II-IV)

The Western blot (also called protein immunoblot) is a commonly used technique to detect specific proteins in tissues or cells extracts. In **Papers II** and **III**, Western blot was performed to evaluate expression levels of BBC3/PUMA (BCL2 binding component 3), TRBP2, DICER or DROSHA in a series of adrenocortical tumors or NCI-H295R cell line. In **Paper IV**, Western blot was used to measure levels of miRNA machinery protein in four *de novo* DLBCL cell lines.

Briefly, about 60µg of cell lysate from cells or tissue samples were separated in Tricine gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes (LC2001; Invitrogen). The membrane was subsequently incubated with a primary antibody against the protein of interest followed by incubation with a secondary antibody, which recognizes a species-specific proportion of the primary antibody. The secondary antibody is linked to a horseradish peroxidase, which, in presence of an appropriate substrate, generates luminescence in proportion to the amount of protein. GAPDH (glyceraldehydes-3-phosphate dehydrogenase) protein was used for normalization and protein levels were quantified on X-ray films from immunoblots using ImageJ software (http://rsb.info.nih.gov/ij/).

3.2.4 Immunohistochemistry (IHC) (Paper IV)

Immunohistochemistry is a technique for detection and localization of proteins in cells of a tissue section using specific antibodies. In **Paper IV**, IHC was performed as previously described (Berglund et al., 2002; Berglund et al., 2005), to evaluate the expression levels of CD10, Bcl-6, IRF-4 and Bcl-2 in 22 *de novo* DLBCLs in order to sub-classify the cases into GC or non-GC phenotype (Hans CP et al., 2003). The expression was considered as positive if >30% of tumor cells showed immunoreactivity for CD10, Bcl-6 or IRF-4 and >50% for Bcl-2.

3.2.5 Transfection experiments in established cell lines (Papers II-IV)

In **Papers II** and **III**, pre-miR Precursor or anti-miR Inhibitors (Applied Biosystems/Ambion) were used for *in vitro* studies to evaluate phenotypic effects or mRNA target identification in NCI-H295R adrenocortical carcinoma cells.

Pre-miR precursors are chemically modified double-stranded RNA molecules which mimic endogeneous mature miRNAs up-regulating their activity. Once introduced in the cells, the special design of these molecules allows the strand with the same sequence of the mature miRNA of interest to be incorporated in the RISC complex, which is responsible for the miRNA activity. On the other hand, anti-miR inhibitors are chemically modified single stranded nucleic acids that can specifically bind and inhibit

expression of endogeneous miRNAs. Specifically, miR inhibitors anti-483-3p or anti-483-5p or miR precursors pre-miR-195 or pre-miR-497 were electroporated in NCI-H295R cells using Nucleofector Technology (Amaxa Biosystems, Gaithersburg, MD). Transfection efficiency was evaluated by measuring the expression levels of *miR-483-3p*, *miR-483-5p*, *miR-195* or *miR-497* using qRT-PCR. Consequences of miRNA expression alteration were evaluated on cell proliferation and apoptosis (**Paper II**).

Further, in **Paper III**, we determined the effects of *miR-195* or *miR-497* over-expression on the expression levels of potential targets such as TRBP2 and DICER.
In **Papers III** and **IV**, NCI-H295R cells were also transfected by electroporation using siRNA molecules (Santa Cruz Biotechnology Inc.). The siRNAs consist of a pool of three 19-25nt long RNA molecules designed to knock down the expression of TRBP2. Our purpose was to inhibit the expression of TRBP2 in NCI-H295R cells (**Paper III**) or in DLBCL cell lines, OCI-Ly-1 and OCI-Ly-3, (**Paper IV**) and to subsequently evaluate whether cell growth and/or apoptosis could be affected.

3.2.6 Quantification of cell proliferation and apoptosis (Papers II-IV)

Malignant cancer cells grow in uncontrolled manner, become resistant to programmed cell death (apoptosis) and acquire the ability to invade adjacent tissues and to metastasize. To evaluate the importance of TRBP2 in tumor development, we investigated the effects of TRBP2 inhibition on cell proliferation and apoptosis in NCI-H295R adrenocortical cancer cell line or in *de novo* DLBCL cells (**Papers III** and **IV**). Further, in **Paper II** we also evaluated the consequences of *miR-483-3p* and *miR-483-5p* inhibition or *miR-195* and *miR-497* over-expression on cell growth and apoptosis.

Cell proliferation was measured using WST-1 (4-(3-(4-iodophenyl)-2-(4 nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate (Roche Applied Science, Mannheim, Germany) colorimetric assay. This is a nonradioactive assay based on the cleavage of tetrazolium salt WST-1 by viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture. Compared to other methods such as MTT (3-4,5-Dimethylthiazol-2-yl)-2-5-diphenyltetrazolium) assay, WST-1 colorimetric assay has many advantages: a) It does not require solubilization steps before the detection; 2) it is more sensitive than MTT; and 3) it is less toxic to the cells.

To perform the proliferation assay, cells were seeded in 96 wells plates and 20 µl of tetrazolium WST-1 was added into 200 µl of cultured cells 48h (for *de novo* DLBCL cells) or 72h (for NCI-H295R cells) after transfection. After incubation at 37°C,

absorbance was determined using a microplate ELISA reader. The measured absorbance directly correlates to the number of viable cells.

For detection of apoptosis, caspase-3 colorimetric assay (Genscript, Poscataway, NJ, USA) was performed. This is a simple method to detect the activity of Caspase-3, which belongs to a family of proteases with an important role in apoptosis. The assay is based on spectrophotometric detection of the chromophore p-nitroaniline (pNA) which is released from the substrate DEVD after cleavage by Caspase-3 (or DEVDase). The yellow color produced by free pNA is proportional to the amount of Caspase-3 activity in the sample analyzed. For detection of apoptosis, lysates containing 50µg protein were mixed with reaction buffer and Caspase-3 substrate for 4h at 37°C. Absorbance was determined using a microplate ELISA reader.

3.2.7 Argonaute 2 (Ago2) immunoprecipitation and analysis of Ago2-associated mRNAs (Paper III)

Protein-RNA interactions play an important structural and regulatory role in the cells and immunoprecipitation (IP) is a valuable method to study these types of interactions.
In **Paper III**, we performed immunoprecipitation of Ago2 in NCI-H295R cells upon over-expression of *miR-195* or *miR-497* to determine whether *TRBP2* and *DICER* were direct targets for these two miRNAs. This protocol is based on the notion that Ago2 complexes can stably associate with miRNA targets (Beitzinger et al., 2007; Karginov et al., 2007). Thus, cells over-expressing *miR-195* or *miR-497* should show an enrichment of *TRBP2* and *DICER* mRNAs in the Ago2 immunoprecipitates in comparison to cells transfected with control molecules. In principle this method consists in combining UV RNA-protein crosslinking with immunoprecipitation using a specific antibody against Ago2 followed by the isolation of the crosslinked RNA. Detection of RNA transcripts can be performed using different techniques such as sequencing, microarray or qRT-PCR.

In our study, *TRBP2* and *DICER* mRNA levels were measured by qRT-PCR and normalized to *18S*. The enrichment of the two mRNAs binding to Ago2 was calculated by dividing the relative amount of mRNA in the IP samples and the amount of mRNA in the corresponding input samples.

3.2.8 Genomic DNA sequencing for mutation screening (Paper III)

Genomic DNA sequencing, using the chain-terminator method (also named Sanger method after its developer Frederick Sanger in the early 1970s), was performed in **Paper III** to identify possible mutations in the *TRBP2* gene.

This method is based on the use of dideoxynucleotides (ddNTPs) in addition to the normal nucleotides (NTPs) present in DNA molecules. ddNTPs basically share the same structure as nucleotides with the exception that they contain a hydrogen group on the 3' carbon instead of a hydroxyl group. This modification prevents the addition of further nucleotides terminating the DNA chain elongation. During the sequencing reaction, the ddNTPs (ddATP, ddCTP, ddGTP and ddTTP) are randomly incorporated in the growing DNA sequence leading to generation of DNA fragments of different length. All DNA fragments are subsequently separated according to size using automated capillary sequencers. The four ddNTPs can be easily detected due to their different absorbance and the sequencing results are displayed as chromatograms.
In this study, we sequenced all coding exons and flanking exon-intron junctions of the *TRBP2* gene in a cohort of 23 ACCs.

3.2.9 TaqMan copy number assay (Paper III)

Copy number variation is associated with genetic diseases such as cancer. In **paper III**, TaqMan copy number assay (Applied Biosystems) was performed to evaluate changes of *TRBP2* copy number in normal adrenal cortices and adrenocortical tumors. In brief, genomic DNA is amplified simultaneously using a TaqMan copy number assay specific to the gene of interest and a TaqMan copy number reference assay (RNase P), which detects a sequence known to be present in only two copies in the genome. The number of copies of the target sequence in each sample is calculated using the comparative C_T method.

3.2.10 Statistical analyses (Papers I-IV)

In all four studies, statistical analysis was performed using Statistica 8.0 (StatSoft, Inc., Tulsa, OK) or MS Office Excel. For miRNA microarray data analysis two statistical methods were applied: Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/Btibs/SAM/) and Prediction Analysis of Microarrays (PAM) (http://www-stat.stanford.edu/Btibs/PAM/). SAM identifies set of genes, which show statistically significant differences in expression between sample groups. Each gene is given a score called false discovery rate (FDR). In our studies, SAM was performed to identify specific miRNAs associated with sample groups, molecular/histopathological and clinical features. PAM is a statistical method for class prediction based on expression data. In our case, PAM was used to determine miRNA classifiers that best could classify sample groups. Unpaired student's *t*-test was used to compare differences in mRNA or miRNA expressions, evaluated by qRT-PCR, between sample groups while paired student's *t*-test was performed to analyze transfection experiments. Pearson's correlation was used to assess correlation between microarray and qRT-PCR results or to evaluate correlation between mRNA and protein

levels. The association between mRNA or miRNA expression and clinical-histopathological parameters was analyzed using χ^2-test. Association between mRNA or miRNA expression and patient outcome was studied using Kaplan-Meier plots, and survival curves for each group were compared using the log-rank test. Overall survival was defined as the period (months) from the time of diagnosis until the end of follow-up or death of the patient. Survival was censored if patients were still alive or died for unrelated reasons. Disease-free survival was the time between the initial diagnosis and the end of follow-up or documented recurrence. Survival was censored if patients did not show any recurrence or died for other causes not related to disease. In all studies, *p*-values < 0.05 were considered as significant and were 2-tailed.

4 RESULTS AND DISCUSSION

4.1 Paper I. MicroRNA expression profiles associated with mutational status and survival in malignant melanoma

Evidence of miRNA expression alteration in cutaneous melanoma was previously reported in a few studies (Gaur et al., 2007; Mueller et al., 2009). However, at the time when we carried out this study, data on miRNAs in melanoma were limited to cell lines. Here, we performed a genome-wide miRNA expression profiling in both clinical material (16 melanoma lymph node metastases) and 21 melanoma cell lines in comparison to cultured melanocytes in order to improve our knowledge of miRNA deregulation in melanoma development and impact on patient survival. Microarray results were subsequently validated in an independent cohort of 16 additional melanoma samples.

We demonstrated that both clinical melanoma samples and cell lines have clearly distinct miRNA expression patterns in comparison to non-neoplastic melanocytes. However, considerable differences in miRNA profiles were observed between clinical samples and cell lines possibly due to effects of culturing conditions or cell type heterogeneity within the clinical material. Nevertheless, cell lines and melanoma samples showed commonly deregulated miRNAs. Among others, we observed a significant under-expression of *miR-211* in both clinical samples and melanoma cell lines compared with melanocytes. Interestingly, *miR-211* seems to be one of the most consistently under-expressed miRNAs in melanoma and recently different studies highlighted its important regulatory role in melanoma invasiveness and metastasis.

Since activating mutations in components of mitogen-activated protein kinase pathway are commonly found in melanoma (Chin et al., 1999; Davies et al., 2002), we additionally aimed to find possible correlations between deregulated miRNA in melanoma and mutations in *BRAF* or *NRAS*. We found that low expression of *miR-193a, miR-338* and *miR-565* was associated with cases carrying $BRAF^{V600E}$ mutation while reduced expression of *miR-663* was associated with *NRAS*-mutated cases (Figure 6). However, we did not identify any miRNA commonly deregulated in *BRAF* and *NRAS* mutated melanomas suggesting that BRAF and NRAS might contribute to melanoma development through regulation of distinct signaling pathways.

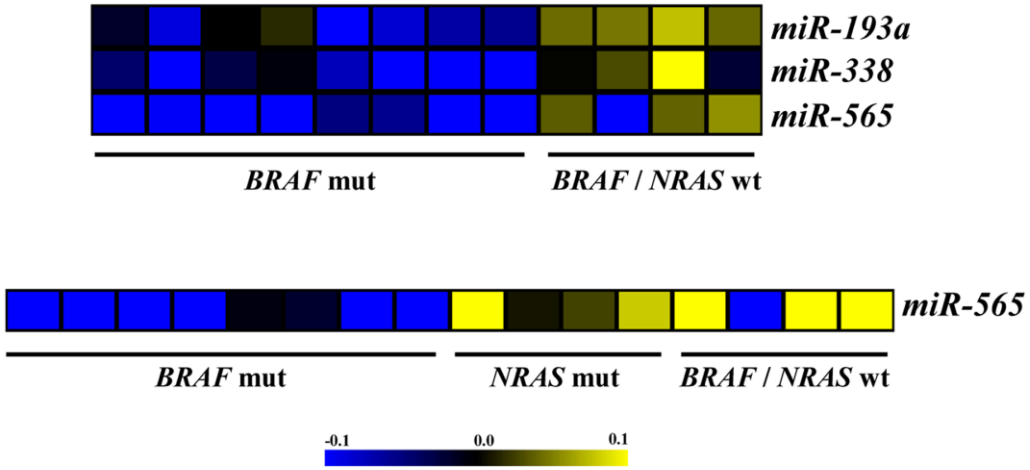


Figure 6. Heat maps showing miRNAs associated to *BRAF* and *NRAS* mutational status. [Modified from (Caramuta et al., 2010)].

In our cohort of samples, *miR-193a* could best discriminate *BRAF*-mutated from wild-type melanomas; interestingly, an association between $BRAF^{V600E}$ mutation and under-expression of *miR-193a* has been also reported in thyroid cancer cells (Cahill et al., 2007). These observations suggest that this miRNA may have a common role in BRAF-associated signaling pathways in different tumor types.

Survival information was available for all the patients included in the study. We then searched for a miRNA signature that could separate melanoma patients with short survival. A univariate analysis was initially performed on a cohort of 16 patients (8 short survival, range 1-12 months and 8 with good prognosis and long survival, range 60-134 months) using microarray data, and subsequently verified by qRT-PCR. Our results showed that low expression of *miR-191* combined with high levels of *miR-193b* were associated with poor survival in melanoma patients. The prognostic value of this miRNA signature was additionally confirmed in an independent cohort of 16 melanoma patients. Association of low *miR-191* and high *miR-193b* expressions with melanoma-specific survival is shown in Figure 7.

In conclusion our study shows a frequent deregulation of miRNA expression in lymph node metastases of malignant melanoma with associations to *BRAF* and *NRAS* mutational status. Moreover, we identified a two-miRNA signature that could predict specific survival in metastatic melanoma patients.

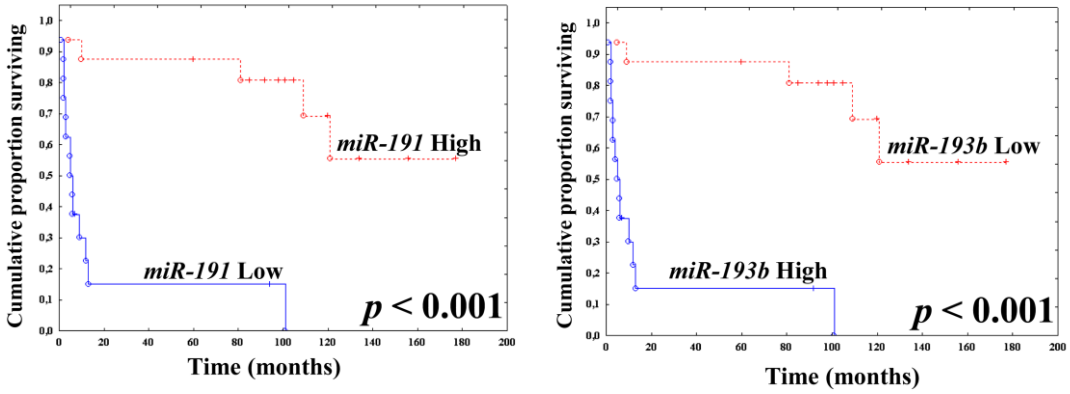


Figure 7. Association between miRNA expression levels and survival in melanoma. Kaplan-Meier plots illustrate the significant associations between low *miR-191* (*left*) and high *miR-193b* (*right*) expression and poor melanoma-specific survival in a cohort of 32 melanoma cases. [Modified from (Caramuta et al., 2010)].

4.2 Paper II. The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma

In this study, we characterized miRNA expression profiles in adrenocortical carcinoma (ACC) in comparison to adenomas and normal adrenal cortex and determined the implication for patient's survival.

Microarray analysis, in a cohort of 48 adrenocortical tumors (16 adenomas and 22 carcinomas) and 4 normal adrenal cortices, showed distinct miRNA expression profiles between ACC and non-carcinoma samples. Among the most significantly deregulated miRNAs, we identified *miR-483-3p*, *miR483-5p*, *miR-497* and *miR-195*. *miR-483-3p* and *miR-483-5p* derive from different arms of the same miRNA precursor while *miR-195* and *miR-497* are generated from the same miRNA cluster and are co-expressed suggesting that they may share common transcriptional regulators and similar biological functions. Over-expression of *miR-483-3p/-5p* and down-regulation of *miR-497-195* cluster in ACC as compared to adenomas or normal cortices was additionally verified in an extended cohort of samples by qRT-PCR. In support of our findings, deregulation of these miRNAs was reported in adrenocortical tumors as well as a variety of other tumors, suggesting that altered expression *miR-483-3p/-5p* and *miR-497-195* cluster may be a common feature in several tumor types (Doghman et al., 2010; Flavin et al., 2009; Lehmann et al., 2010; Patterson et al., 2011; Soon et al., 2009).

Beside the association with expression levels, we also investigated the biological role of these deregulated miRNA in the pathogenesis of ACC. We showed that ectopic expression of *miR-195* or *miR-497* and inhibition of *miR-483-3p* or *miR-483-5p* could significantly reduce cell growth in NCI-H295R human ACC cells. In addition, we observed that over-expression of *miR-195* or *miR-497* and reduced expression of *miR-483-3p*, but not *miR-483-5p*, promoted programmed cell death. Interestingly, effect on cell proliferation and apoptosis, upon repression of *miR-483-3p* expression, was previously shown in hepatocarcinoma cells (Veronese et al., 2010). In the same study, Veronese and colleagues identified BBC3/PUMA, a tumor suppressor involved in apoptosis regulation, as a *miR-483-3p* target. Notably, we showed a significant inverse correlation between *miR-483-3p* and PUMA expressions in adrenocortical tumors suggesting a possible miRNA-mRNA target relationship also in this tumor entity (Figure 8).

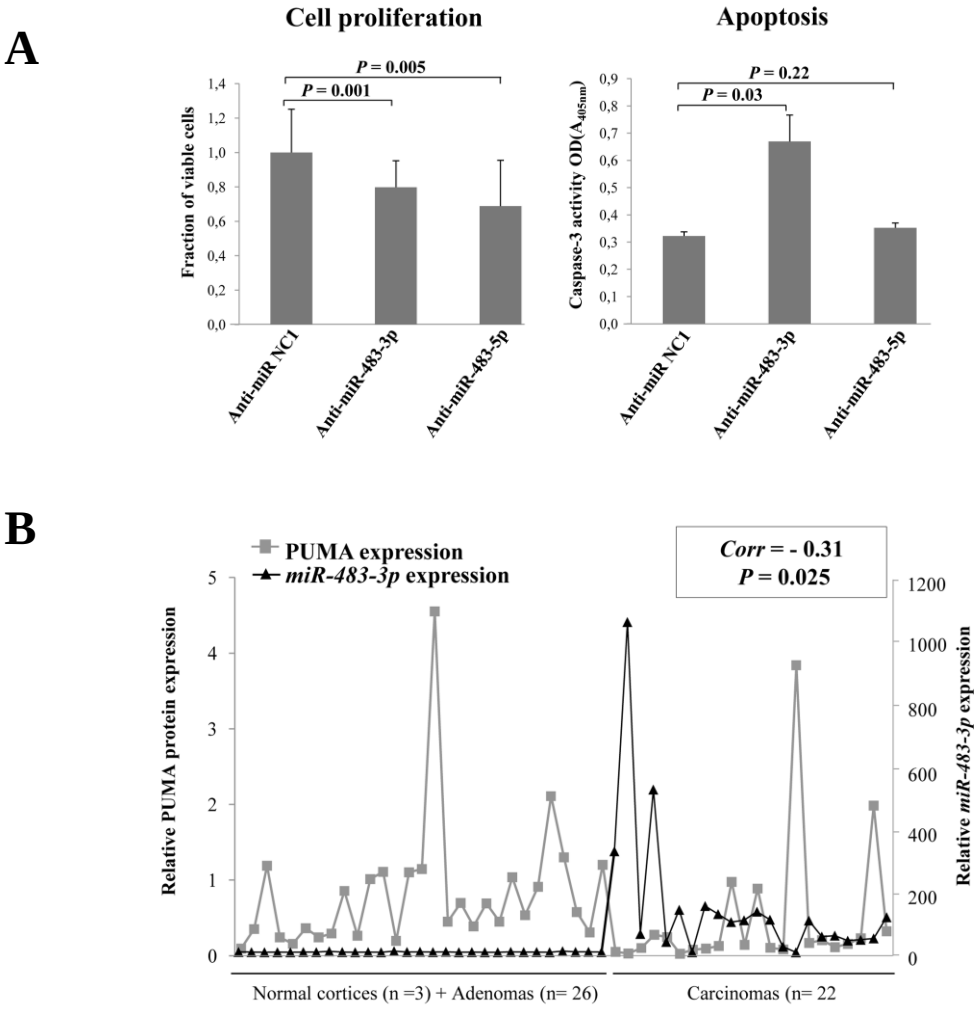


Figure 8. Role of *miR-483* in ACC. (A) Effect on cell proliferation and apoptosis upon inhibition of *miR-483* expression. (B) Correlation between expression of *miR-483-3p* and its putative target PUMA. [Modified from (Özata et al., 2011)].

In this study, we also demonstrated that miRNA expression profiles could sub-classify ACCs according to their clinical outcome. Specifically, using a classifier of 11 miRNAs identified by SAM survival analysis, the 25 ACC cases analyzed were clustered in three distinct groups, of which one of them included most of the ACC cases who died of disease and was associated with poorer survival. The prognostic value of these miRNAs was verified evaluating their expression by qRT-PCR. Kaplan Meier survival curves combined with log-rank test analysis confirmed a significant association of high expression of three miRNAs (*miR-503*, *miR-1202* and *miR-1275*) with poorer survival (Figure 9).

In conclusion, we found additional miRNAs associated with ACC and partly elucidated the functional role of *miR-483*, *miR-195* and *miR-497* in the pathogenesis of ACC. In addition, we identified novel miRNAs associated with poor survival in ACC which may have an important role in patient's management and choice of therapeutic strategy.

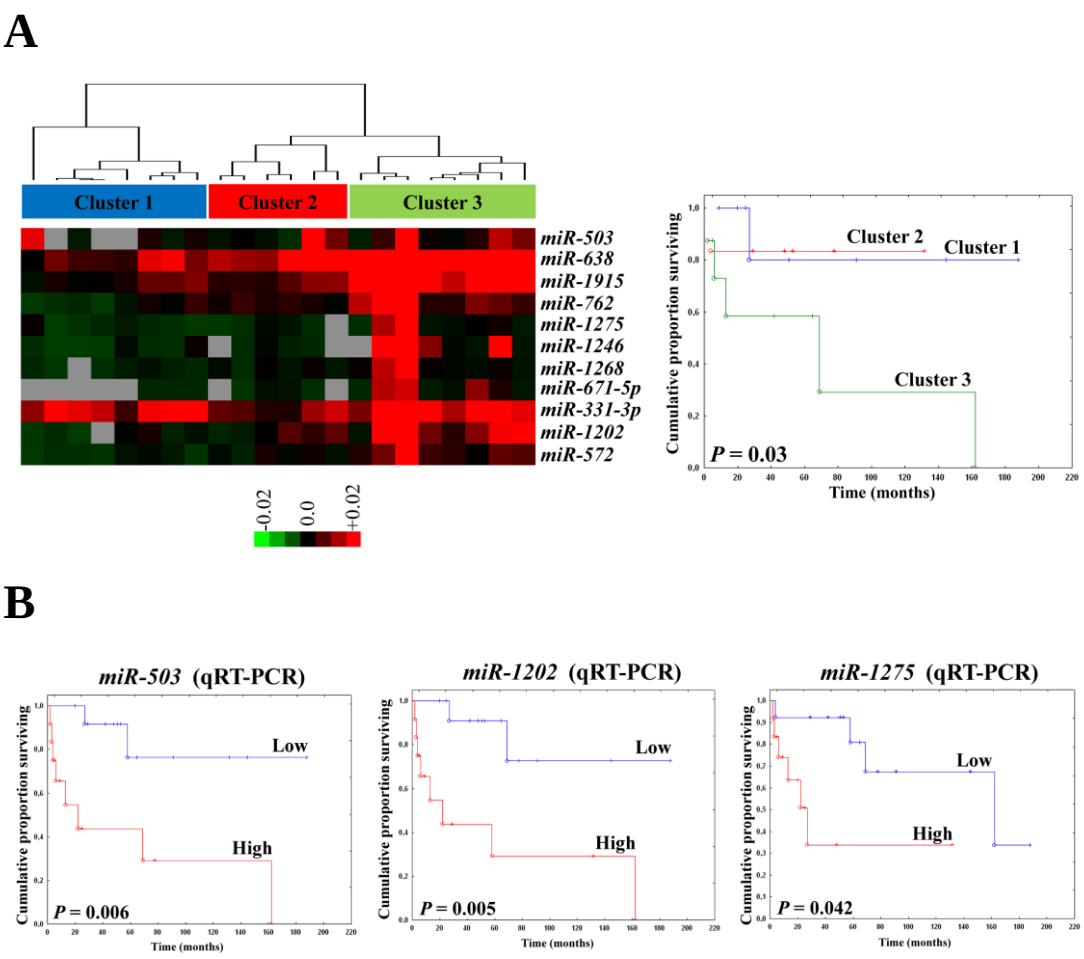


Figure 9. miRNAs associated with survival in carcinoma patients. (A) Identification of 3 clusters based on miRNA expression and subsequent Kaplan-Meier analysis for cases in Cluster 1, Cluster 2 and Cluster 3. (B) Kaplan-Meier curves for *miR-503*, *miR-1202* and *miR-1275* based on qRT-PCR results. [Modified from (Özata et al., 2011)].

4.3 Paper III. Clinical and functional impact of TRBP2 over-expression in adrenocortical carcinoma

We, and others, have previously shown the diagnostic and prognostic impact and functional role of deregulated miRNAs in adrenocortical tumors. In addition, recent studies have also demonstrated frequent alteration of miRNA processing factors, required for miRNA maturation, in cancer and shown their important role in development and progression of different tumor types.

Here, we investigated the expression of miRNA machinery's core components in a cohort of 73 adrenocortical tumors (43 adenomas and 30 ACCs) and 9 normal adrenal gland cortices using qRT-PCR and Western blot approaches. We observed a significant over-expression of TRBP2, DICER and DROSHA, both at mRNA and protein levels, in ACC cases as compared to adenomas and normal tissues (Figure 10). However, while increased expression of TRBP2, DICER and DROSHA was found in some tumor types, a few studies showed low expression of these miRNA machinery components in other human malignancies such as ovarian, lung and gastric cancers (Chiosea et al., 2006; Fu et al., 2010; Karube et al., 2005; Melo et al., 2009; Merritt et al., 2008; Muralidhar et al., 2007). These observations may suggest a tissue-specificity deregulation of TRBP2, DICER and DROSHA, implying a potential dual role as tumor suppressors or oncogenes in different human cancers.

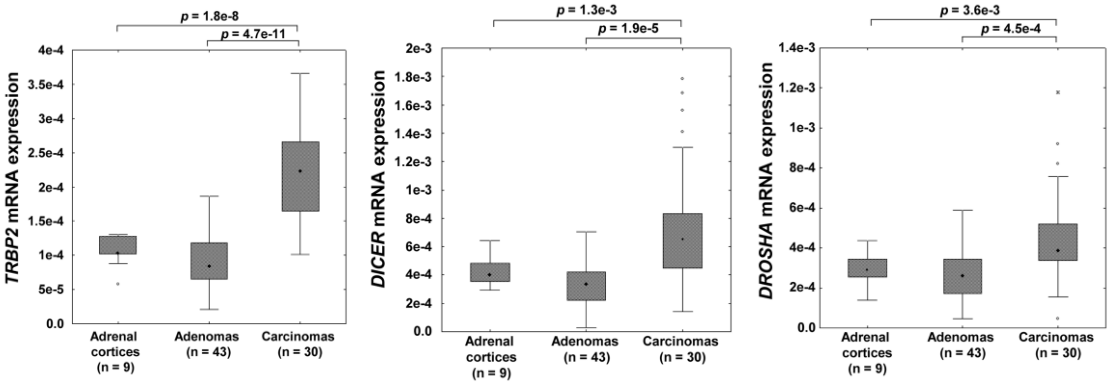


Figure 10. Box plots showing relative mRNA expression levels for *TRBP2*, *DICER*, and *DROSHA* in adrenocortical tumors and adrenal cortices.

A major challenge in diagnosis of adrencortical tumors is to distinguish between localized carcinomas and tumors that are simply adenomas. Here, in order to evaluate the prognostic value of *TRBP2*, *DICER* and *DROSHA* in adrenocortical tumors we performed Kaplan-Meier analysis of disease-free survival on a cohort of 45 non-

metastatic adrenocortical tumors. Our results demonstrated that, among the three genes analyzed, *TRBP2* was the best predictor of ACC (Figure 11).
Thus, expression of *TRBP2* may be of clinical relevance in addition to other clinical tools currently in use for management of adrenocortical carcinomas.

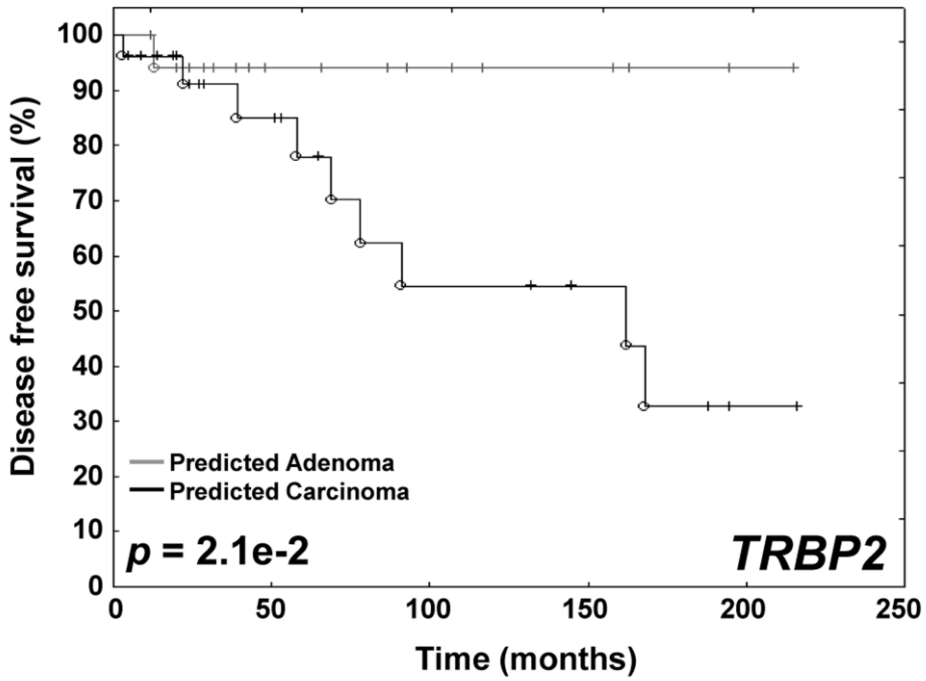


Figure 11. Prediction of carcinomas based on the mRNA expression levels of *TRBP2*. The Kaplan-Meier curves separate the patients according to the expression levels of the molecular predictors *TRBP2*.

Moreover, in agreement with the increased expression in ACC patients, we showed that repression of TRBP2 levels affected cell growth and promoted apoptosis in NCI-H295R cell line. These data further suggest the oncogenic activity of TRBP2 and its possible role in the pathogenesis of ACC.

Beside its biological function, we also unraveled possible molecular mechanisms that regulates TRBP2 expression in ACC. Copy number gain, analyzed using Taqman copy number assay, was observed in 57% of the ACC cases suggesting that copy number abnormality may be partly responsible for the TRBP2 over-expression. In addition, we demonstrated that ectopic expression of *miR-195* or *miR-497* can modulate TRBP2 expression in NCI-H295R cells. In our previous study (Paper II) we have shown that *miR-195* and *miR-497* can regulate cell growth and apoptosis in ACC cells. Taken together, our findings may suggest phenotypic effect observed in adrenocortical cancer

cell line upon alteration of *miR-195* and *miR-497* expression might be mediated through TRBP2 down-regulation. However, gene mutations were not observed suggesting that this is not an additional mechanism of TRBP2 expression regulation.

In conclusion, we showed over-expression of TRBP2 as a frequent event in ACC and demonstrated its functional role in pathogenesis of this tumor type. Further, we found that *TRBP2* mRNA expression is a valuable predictor of malignancy in adrenocortical tumors demonstrating its relevance as a potential novel biomarker in the prognosis of ACC.

4.4 Paper IV. Role of microRNAs and microRNA machinery in the pathogenesis of diffuse large B-cell lymphomas

Diffuse large B-cell lymphoma (DLCBL) is a type of B-cell lymphoma characterized by a highly heterogeneous genetic background. miRNA alterations in DLCBL, has been previously reported (Lawrie et al., 2009; Lawrie et al., 2007; Roehle et al., 2008). However, the role and the clinical impact of miRNAs and their processing factors in this type of lymphoma are still not fully understood.

In this study we analyzed, using microarray and qRT-PCR approaches, miRNAs and their processing factors expressions in a cohort of 75 DLBCLs (56 *de novo* and 19 transformed) and 10 reactive lymph nodes used as references of non-neoplastic lymphatic tissue (LN). Our results show clearly different miRNA patterns between DLBCLs (*de novo* and transformed) and LNs (Figure 12). Even though the two tumor subtypes showed specific differentially expressed miRNAs in comparison to LNs, most of miRNAs were commonly deregulated in both *de novo* and transformed DLBCLs suggesting that the two tumor types may employ partly overlapping regulatory pathways. In addition, we identified a classifier of 11 miRNAs that could distinguish the *de novo* from transformed cases.

Given the genetic variation between DLBCLs, we attempted to find deregulated miRNAs that could characterize different subtypes among the *de novo* cases. We first identified a set of six miRNAs associated with GCB and non-GCB immunophenotypes. Concordant with previous studies, *miR-155* and *miR-146a* were over-expressed in non-GCB cases (Lawrie et al., 2007; Malumbres et al., 2009; Roehle et al., 2008). However, deregulation of the other four miRNAs was not previously reported: discrepancies may be due to interpretations of immunohistochemical staining to distinguish GCB and non-GCB cases, different technical approaches adopted in the studies or the use of cell line instead of clinical samples to identify miRNA signatures. Further, we observed an inverse correlation between altered miRNAs and expression of Bcl-6 and IRF-4, which

are commonly deregulated in DLBCL and have a prognostic impact in this type of lymphoma. We also found that over-expression of *miR-494* was associated with DLBCL cases in advance stages (III-IV) of disease. Even though little is known about the function of this miRNA in lymphomas, increased expression of *miR-494* and its potential role in tumor development has been reported in other cancer types (Arribas et al., 2012; Liu et al., 2010; Wang et al., 2010).

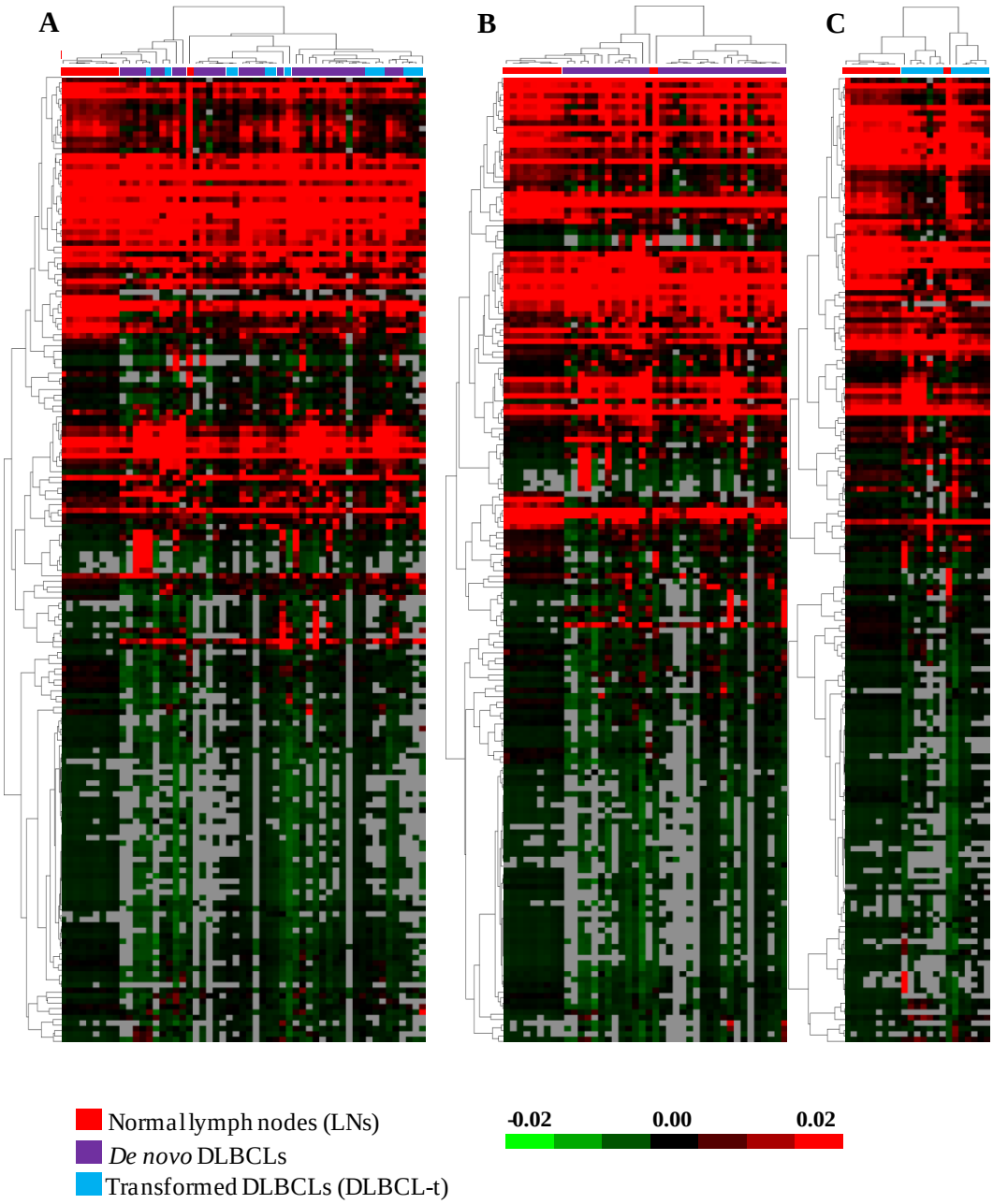


Figure 12. The heat maps show unsupervised clustering of miRNA expression profiling in LNs and DLBCLs (A) as well as LNs and *de novo* DLBCLs only (B) or LNs and transformed DLBCL only (C). The several distinct cluster may reflect the highly heterogeneity that characterize DLBCLs.

Beside the analysis of global miRNA expression, we investigated miRNA machinery deregulation in DLBCL in the attempt to understand the possible role in lymphomagenesis. We observed a significant over-expression of *TRBP2* in *de novo* DLBCLs as compared to LNs and decreased expression of *DROSHA*, *DICER*, *TRBP2* and *PACT* in transformed DLBCLs v.s. *de novo* cases. We then demonstrated that altered expression of miRNA machinery factors could affect the processing efficiency of *miR-155* and *miR-146a*, explaining the observed over-expression of these miRNA in *de novo* DLBCLs (Figure 13). Moreover, we unraveled the tumorigenic potential of TRBP2 in DLBCL showing that inhibition of TRBP2 expression in lymphoma cell lines could affect both proliferation and apoptosis.

In conclusion, our results underline the importance of miRNAs as molecular markers to sub-classify DLBCL tumors. Further, we showed the possible implications of miRNA processing factors deregulation in the development of DLBCLs.

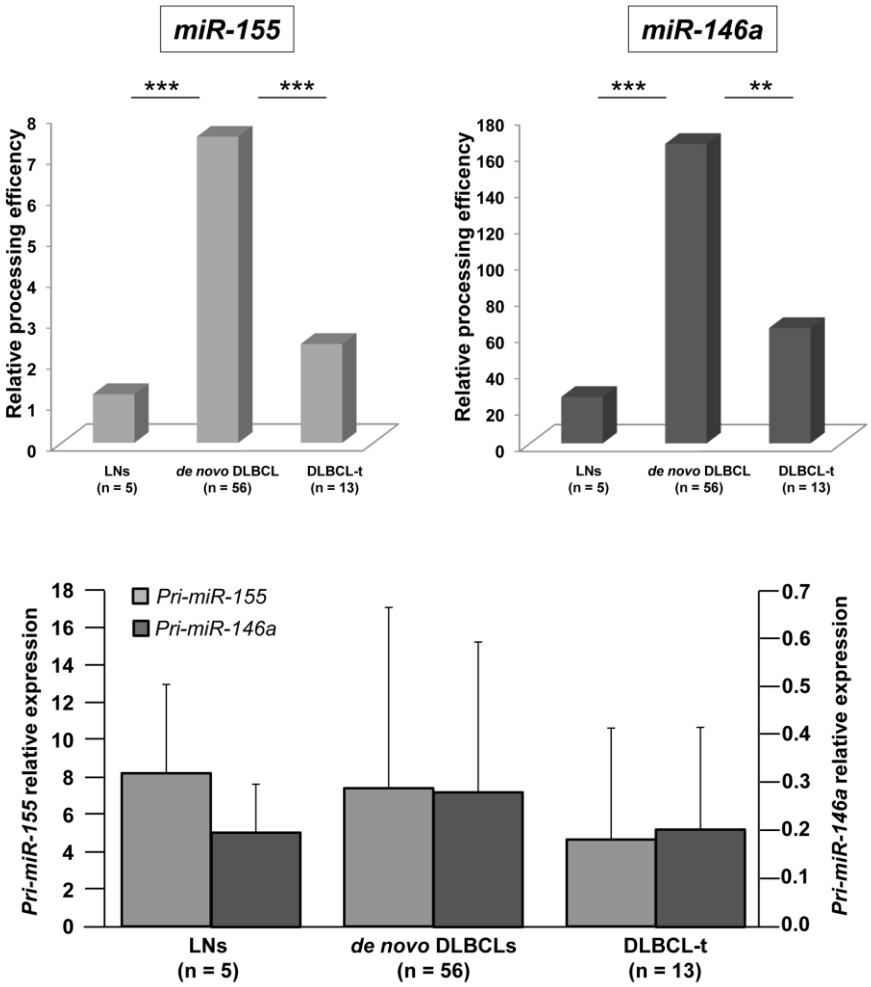


Figure 13. Histograms show a significant higher miRNA processing efficiency in *de novo* DLBCLs as compared to transformed DLBCLs and LNs (top), while the levels of pri-miRNA are similar between groups (bottom). **p<0.01; ***p<0.001.

5 CONCLUDING REMARKS

The miRNA field is in a continuous growth and an increasing number of studies are constantly underlining the crucial importance of these small non-coding RNAs in the pathogenesis of different tumor types. Despite all the achievements, our knowledge of the biological role of these molecules in tumor development and progression is still in its infancy. In this thesis work we attempted to improve our understanding of the clinical and functional impact of miRNAs in three different cancer types, *e.g*. metastatic melanoma, adrenocortical carcinoma and diffuse large B-cell lymphoma. According to our findings we can conclude that:

1. miRNA expression deregulation is a frequent event in different tumor types such as metastatic melanoma, adrenocortical carcinoma (ACC) and diffuse large B-cell lymphoma (DLBCL) (**Papers I**, **II** and **IV**). In addition, using DLBCL as tumor-model, we confirmed the potential of miRNAs as molecular biomarkers to classify different tumor sub-types (**Papers IV**).

2. We demonstrated the prognostic value of miRNAs in human cancer, identifying miRNA signatures associated with poor prognosis in patients with metastatic melanoma and ACC (**Papers I** and **II**). In addition, we partly elucidated the functional role of *miR-483*, *miR-195* and *miR-497* in the pathogenesis of ACC and showed the potential involvement of the pro-apoptotic factor PUMA (a target of *miR-483-3p*) in adrenocortical tumors.

3. Beside miRNA expression alteration, we reported deregulation of miRNA processing factors in ACC and DLBCL tumors. We also demonstrated that abnormal expression levels of miRNA machinery genes can affect miRNA biogenesis. Further, we showed that over-expression of TRBP2 was a common event in both ACC and *de novo* DLBCL tumors and we unraveled its potential oncogenic role in the pathogenesis of both tumor types. Additionally, we proposed possible regulatory mechanisms (*e.g* copy number variation and miRNA-mediated post-transcriptional regulation) that could lead to TRBP2 over-expression in ACC (**Papers III** and **IV**).

4. We showed that *TRBP2* is a useful predictor of malignancy to reliably discriminate adrenocortical carcinomas from adenoma tumors. These findings suggest that TRBP2 might be used as a novel molecular marker in combination with other tools currently in use in clinical practice for management of adrenocortical carcinomas (**Paper III**). Given the role of TRBP2 as oncogene and its prognostic value in ACC, we also speculate that deeper understanding of the molecular mechanisms involved in the over-expression of TRBP2 in ACC may lead to development of new and more targeted therapies.

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