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1. INTRODUCTION

1.1 microRNAs

MicroRNAs (miRNAs) are a class of endogenous non-protein-coding small RNAs that are evolutionarily conserved and widely distributed among species (*June et al., 2011*).

The first microRNA was identified by Lee et al. and Wightman et al. simultaneously that discovered an approximately 22-nucleotide, non-protein-coding small RNA in *C. elegans* in 1993 (*Reinhart et al.,2000*) : *lin-4,* a gene known to control the timing of *C. elegans* larval development, does not code for a protein but instead produces a pair of small RNAs (*Lee et al., 1993*). One RNA is approximately 22 nt in length, and the other is approximately 61 nt: the longer one was predicted to fold into a stem loop proposed to be the precursor of the shorter one. Researchers noticed that these *lin-4* RNAs had antisense complementarity to multiple sites in the 3′ UTR of the *lin-14* gene (*Lee et al., 1993; Wightman et al., 1993*). This complementarity fell in a region of the 3′ UTR previously proposed to mediate the repression of the *lin-14* by the *lin-4* gene product (*Wightman et al., 1991*). These complementarity sites for regulation of *lin-14* by the *lin-4* showed also that this regulation substantially reduced the amount of LIN-14 protein without noticeable change in levels of *lin-14* mRNA. Together, these discoveries supported a model in which the *lin-4* RNAs pairing to the *lin-14* 3′ UTR to specify translational repression of the *lin-14* message is part of the regulatory pathway that triggers the transition from cell divisions of the first larval stage to those of second (*Lee et al., 1993; Wightman et al., 1993*).

In 2000, another non-coding small RNA, *let-7*, was discovered to regulate nematode development. Similarly to *lin-4, let-7* is an endogenous non-coding RNA with a length of 22 nt that regulates the transformation of nematode from late-stage larva to adult nematode (*Reinhart et al.,2000*).

The majority of human miRNA-coding genes are located in the introns, non-coding exons, or 3′ untranslated regions (3′ UTRs) of protein-coding genes (*Rodriguez et al., 2004*). A small number of miRNA genes are located within non coding transcripts in the genome (*Bartel, 2004*).

miRNA genes usually occur in clusters. Multiple miRNA genes are transcribed into a common precursor miRNA (pri-miRNA) that is processed into multiple mature miRNAs.

miRNA expression exhibits temporal and tissue-specific patterns. For example *lin-4* is mainly expressed at the first and second larval stage in *C.elegans*, whereas *let-7* is primarily expressed at the third and fourth larval stages and the adult stage. In human, miR-1 is only expressed in myocardial and skeletal muscle cells, whereas miR-122 is only expressed in hepatocytes.

miRNAs are highly conserved during evolution. The broad distribution and evolutionary conservation of miRNAs suggest their indispensable regulatory functions in vital biological processes (*June T. et al,2011*).

1.2 THE BIOLOGY OF MICRORNA

1.2.1 Biogenesis

In mammals, the majority of miRNAs are located within introns of either protein-coding or non-coding host genes, while others, depending on the occurrence of alternative splicing, are present either in an exon or an intron. A significant number of miRNAs are also assembled in clusters in which two or three miRNAs are generated from a common parent mRNA**.** Many of discovered miRNAs are specifically expressed as tissue-stage and/or developmental stage miRNAs, and this can be attributed to regulatory sequences present in their promoters (*Babak P. et al., 2004; Barad O. et al., 2004; Zhao Y. et al., 2005; O'donnel K.A. et al., 2005)*.

Two converging pathways have been discovered for the biogenesis of miRs in animals (Figure 1). In the first, the canonical pathway, transcription of miR genes yields transcripts, termed primary miRs (pri-miRs), that are up to several thousands of bases long (*Cai X. et al. 2004*). pri-miRs have a characteristic hairpin morphology, comprising a loop and an imperfectly paired stem incorporating the mature miR sequence on one of the strands near the loop. Transcription of miR genes is polymerase II-dependent (some miRs that are interspersed among repetitive DNA elements, however, are polymerase III dependent) (*Borchert GM. et al., 2006*) and is regulated by transcription factors.

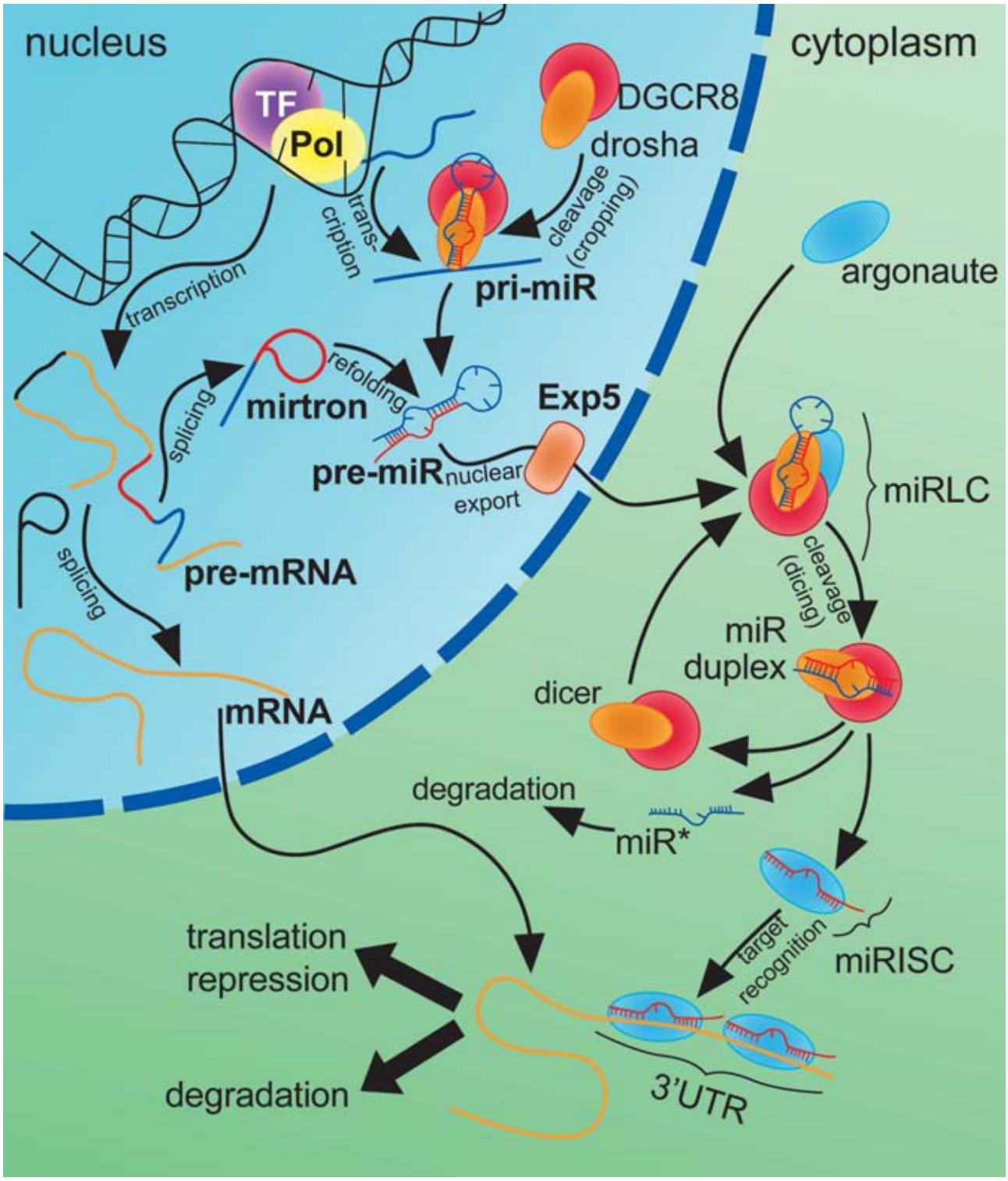


Figure 1: Schematic of microRNA biogenesis and action.

Many miR genes are polycistronic in that they encode two or more stem-loops that can each be processed into distinct mature miRs (*Bartel, 2004*). As the pri-miR is transcribed, a nuclear enzyme called Drosha (bound to a cofactor, the DiGeorge syndrome critical region 8 -DGCR8-) processes the pri-miRNA by cropping the distal stem portion. Important for their recognition in later processing, cleavage by Drosha introduces staggered cuts on each side of the RNA stem, resulting

in a 5′ phosphate and a two-nucleotide overhang at the 3′ end. This produces a shorter hairpin, called precursor miR (pre-miR) (*Lee et al., 2003*). The pre-miRs can then be transported to the cytoplasmic compartment of the cell by exportin-5 (*Yi R.et al., 2003*). Final processing is carried out by the miR-induced silencing complex (miRISC)-loading complex (miRLC) (*Maniataki E. et al., 2005*). The miRLC is an agglomeration of proteins that: 1. removes the loop portion of the pre-miR (by an enzyme called Dicer) (*Hutvagner G. et al.,* 2001) to form a double-stranded miR duplex, 2. strips away what is called the passenger (or miRNA*) strand from the duplex to leave a mature miR, and 3. transfers the mature miR from Dicer to another protein of the miRLC, called Argonaute (Ago) (*Hutvagner G. et al.,* 2008). The effector of miRNA-mediated RNA silencing is the miRISC, which is composed of the mature miR attached to an Ago protein and a GW182 protein (*Eulalio A. et al., 2009*).

In 2007, a second pathway was identified in which the miRs (termed mirtrons) derive from introns that are the correct size to form pre-miRs directly (*Ruby J.G. et al., 2007 Okamura K. et al., 2007*). The mirtrons are spliced out of their host gene to form looped intermediates (or lariats) that are then debranched and refolded into the usual stem-loop structure of pre-miRs; mirtrons, therefore, bypass the Drosha processing step.

From here, mirtrons access the canonical biogenesis pathway described above. To date, only a small number of mirtrons have been found in primates (*Berezikov E. et al., 2007*). However, some mammalian mirtrons might have a longer 5′-tail (tailed mirtrons), so the introns that potentially contain this type of miR might be more numerous than first thought (*Babiarz J.E. et al., 2008*).

1.2.2 Mechanisms of action

The effects of miRNAs are produced mainly within the cytoplasm of the cells through their base pairing with complementary sequences present at the 3′- untranslated region (UTR) of target mRNAs (Fig. 2, right box). The binding specificity between miRNAs and these mRNAs is dictated by only 6-7 out of the approximately 22nt that compose a miRNA. This sequence is called the *seed sequence* and is located at the 5′-end of the miRNA molecule, often in multiple copies (*Pillai et al., 2005*). The rest of molecule usually binds with only partial complementarity, producing characteristic mismatch bulges, especially in the central region and to a lesser extent at the 3′-end. Occasionally, the pairing of the miRNA seed can be marginally suboptimal, but miRNA-mRNA annealing can be stabilized by a higher degree of complementarity at the 3′-end. Of relevance are the thermodynamic properties of UTR target sites (*Zhao et al., 2005*). In fact, while an unstructured secondary configuration located in an accessible region may facilitate miRNA pairing, a more stable and complex structure may interfere with the binding of miRNAs even with high sequence

complementarity. However, in particular cellular conditions, an unfolding of these stable secondary structures might be promoted, thus rendering the same site accessible. RNA-binding proteins or miRNAs could, therefore, function cooperatively to alter the complexity of regions by binding specific neighboring of other miRNAs. This might introduce another level of miRNA target selection (*Catalucci et al., 2008*).

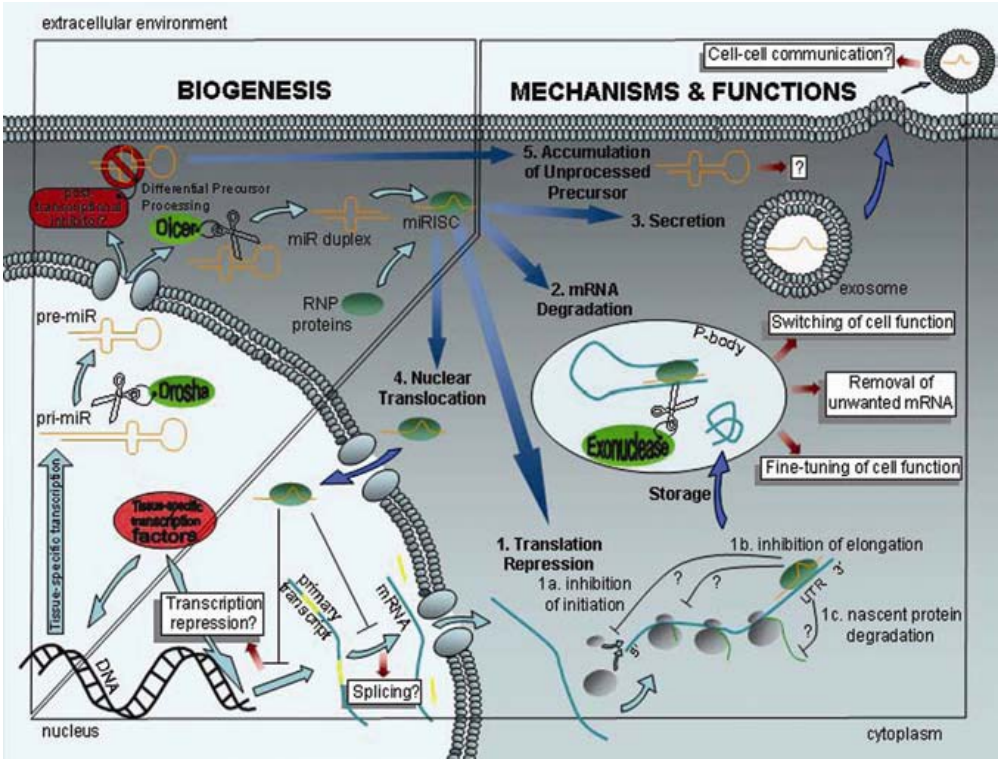


Figure 2: Schematic representation of miRNA biogenesis (left box) and mechanisms (right box).

Differences in pairing seem also important for the type of post-translational control that would be produced. When binding is partial, miRNAs are responsible for the reduced translation of targeted mRNAs (Fig. 2, 1 Translation Repression). The exact mechanism of action is still not clear since steps both before and after translation initiation have been reported as the point of repression. In fact, inhibition of initiation factor (IF) 4E-dependent initiation, (*Humphreys et al, 2005 and olsen et al., 1999*), elongation (*Lee at al., 1993; Wightman et al., 1993 and Maroney et al., 2006*) and degradation of nascent protein (*Nottrott et al., 2006 and Tang, 2005*), among others, have been reported (Fig. 2, 1a-c).

When miRNAs bind with precise complementarity to target mRNAs, they behave similarly to siRNAs and signal for mRNA degradation (*Zhao et al., 2005; Schmitter et al., 2006*). In contrast, because miRNAs are transported into the cytoplasm, miRNA-mediated mRNA degradation occurs not via an siRNA-like mechanism of endonucleolytic cleavage but rather through the normal pathway of deadenylation followed by decapping and subsequent degradation by exonuclease

activity (Fig.2, 2. mRNA Degradation). It has also been shown that miRISC components localize to structures called *processing bodies* (P-bodies) (*Li uet al., 2005*). These are cytoplasmic foci containing enzymes important in the normal pathway of mRNA degradation.Within these P-bodies, translationally repressed mRNA is either sequestered in storage structures or processed for degradation (Fig. 2).

MicroRNAs have been recently implicated in a cell to cell communication mechanism whereby cells are involved in an exchange of genetic material (*Valadi et al., 2007*). Many types of cells are known to release proteins into the extracellular environment via exosomes.These structures have now been demonstrated to contain molecules of RNA, including miRNAs (Fig. 2, 3. Secretion). This finding has raised the exciting possibility that cells can modify gene expression not only of other nearby cells but also, if released into the circulation, of cells at distant sites, with miRNAs acting akin to hormones.

All the above described functions depend on post-translational repression (either via reduced translation efficiency or degradation of the targeted mRNA), thought to be the sole mechanism through which miRNAs act. However, this view has been recently challenged. Although relatively small in size, miRNAs can still contain specific sequences at the 3′-end that are responsible for controlling their post-transcriptional behavior. In fact, miRNAs, such as miRNA-29b, may contain a distinctive 3′-hexanucleotide terminal motif responsible for its relocation back into the nucleus during the cycling phase of cells (Fig. 2, 4. Nuclear Translocation) (*Hwang et al., 2007*). The exact function of nuclear relocated miRNAs, however, is not yet understood, but it has been speculated to involve either transcriptional control or splicing regulation. Furthermore, differential precursor processing has been documented for some miRNAs, such as miRNA-138, that, while having a ubiquitous expression of the pre miRNA, are characterized by selective maturation occurring in certain cell types or at a particular developmental stage (*Obernosterer et al 2006*). Evidence points to the presence of an inhibitor that binds to the pre-miRNA and that prevents pre-miRNA processing by Dicer. Accumulation of the pre-miRNA within the cytoplasm might not only represent an additional level of control of miRNA expression (thought to occur primarily at the transcriptional level) but it also suggests the presence of a novel function for miRNA precursors (Fig. 2, 5. Accumulation of Unprocessed Precursor).

1.3 ROLE AND EXPRESSION OF MICRORNAs

Studies in human cells and model organisms have begun to reveal the mechanisms of microRNA activity, and the wide range of normal physiological functions they influence. Their alteration in pathologic states from cancer to cardiovascular disease is also increasingly clear (*Boyd 2008*).

1.3.1 Profiles of miRNAs in normal human tissue and cells

About one-third of microRNAs show substantial tissue specificity, while the others may vary in expression level but are not particulary tissue or cell type-specific. Among the most specific miRNA expression patterns are that of miR-122 in the liver, miR-375 in pancreatic isle tissue, miR-142 and miR-223 in the ematopoietic system, and miR-1 and miR-133 in muscle (*Landgraf et al., 2007; Poy et al., 2004; Sempere et al., 2004*). The utility of some tissue-specific microRNAs for identifying the tissue of origin of poorly differentiated metastases of unknown primary tumors in human patients has been demonstrated (*Lu et al., 2005*). Some studies on mouse model knockout for different microRNAs have shown as some animal tissue and cell lineage may be more dependent on microRNA regulation than others (*Boyd et al., 2008*).

1.3.2 Stem cells biology

A small mumber of microRNAs are expressed mainly in embryonic stem cells (ES), and attention has naturally focused on their roles in maintaining the pluripotent undifferentiated state and other characteristics of ES cells (*Houbaviy et al., 2003; Suh et al., 2004*). Some evidence for such roles comes from the poor proliferation and differentiation potential of Dicer-null murine ES cells, and from the small number of germline stem cells produced in Drosophila mutants of Dicer1 or its accompanying RNA-binding protein, Loquacious (*Hatfield et al., 2005; Forstemann et al., 2005; Murhison et al., 2005; Park et al., 2007*). In contrast, mouse ES cells null for the microprocessor complex member DGCR8, which is also required for microRNA biogenesis, showed a less severe impairment of division in culture while still being unable to differentiate normally into other cell types when appropriately stimulated (*Wang et al., 2007*). Together, the mammalian ES cell studies of Dicer and DGCR8 mutants shows these proteins having overlapping but not identical functions, suggesting that one or both may be involved in other pathways besides the microRNA pathway as it is currently understood (*Boyd et al., 2008*).

1.3.3 Germline

Tissue-specific ablation of Dicer gene in mouse oocytes results in abnormalities culminating in meiosis I arrest with severe defects in meiotic spindle formation and chromosome organization. In addition, mRNAs that are normally degraded in meiotic maturation in oocytes, and that have predicted target sites for oocyte-expressed microRNAs, were present at elevated levels in Dicer-null oocytes, suggesting that the microRNAs may partecipate in specifying their destruction (*Murchison et al., 2007*).

1.3.4 Cardiac Muscle

MicroRNAs are estimated to comprise at least 1% of animal genes (*Berezikov et al., 2005*) and regulate 30% of the human genome (*Lewis et al., 2005*), making them one of the most abundant classes of regulators (*Stark et al., 2005*), with a pattern of expression that is often perturbed in disease states (*Lu et al., 2005; Care et al., 2007; Alvarez et al., 2005*). A large array of miRNAs can be found within tissues of an organism, and at least one miRNA is specifically expressed per tissue (*Wienholds et al., 2005*). In muscle, for example, miRNA-133 has been found to be preferentially expressed. Other miRNAs, such as miRNA-1, have also been found to be muscle specific (*Sempere et al., 2004; Basjerville et al., 2005*). To date, only miRNA-208a has been found to be purely cardiac specific.

The miRNA-1 family is one of the most highly conserved and consists of miRNA-206 (which is not expressed in cardiac muscle) and two closely related transcripts, miRNA-1-1 and miRNA-1-2 (*Wienholds et al., 2005; Brennecke et al., 2005; Sokol et al., 2005*). The miRNA-1 family is found as part of a polycistronic unit that is transcribed together with components of the miRNA-133 family, comprised of miRNA-133a- 1, miRNA-133a-2, and miRNA133-b paralogs. Chromosome 2 contains the miRNA-1-1/miRNA-133a-2 intergenic bicistron, while miRNA-206/miRNA-133b (also intergenic) is found on chromosome 1. On the other hand, miRNA-1-2/miRNA-133a-1 is intronic and is located on chromosome 18; these miRNAs are found on the opposite strand of the nonmuscle-specific protein-encoding gene, Mindbomb (Mib1), between exons 12 and 13, demonstrating the complex characteristics of miRNA genetics (*Rao et al., 2006*).

At the regulatory level, mammalian cardiac miRNA-1 is controlled by the serum response factor (SRF), which recruits a coactivator, myocardin, to muscle-specific genes that control differentiation (*Zhao et al., 2005*). This is slightly different from that occurring in skeletal muscle where miRNA-1 expression requires myogenic transcription factors, such as myogenic differentiation 1 (MyoD), myocyte enhancer factor 2, and myogenin. In addition, the presence of putative transcription factor binding sites in between miRNA-1-1 and miRNA-133a-2 suggests the possibility that the individual

miRNAs contained in the polycistronic unit may be independently regulated (*Rao et al., 2006*). Concurrently, similar transcriptional control has been shown for skeletal muscle-specific expression of miRNA-133 (*Chen et al. 2006*). However, in cardiac muscles, where expression of MyoD and myogenin is not observed, modulation of miRNA- 133 levels is mainly regulated by SRF, as shown for miRNA-1. In addition, miRNA-133 has been reported to repress SRF, suggesting a possible regulatory loop (*Chen et al. 2006*).

1.3.5 Hematopoiesis and Immunity

Normal hematopoiesis and immunity are guided by microRNA-mediated gene regulation. miR-181 is expressed in hematopoietic tissues, and at lower levels in several other tissues such as muscle (*Naguibneva et al 2006*). Overexpression of miR-181 in murine hematopoietic progenitor cells increases the relative proportion of B cells to T cells in peripheral blood; interpretation of the mechanisms of this effect is complicated by the role played by this microRNA in modulating thymocyte T cell receptor signalling and clonal selection, but is clearly influences the final balance of the two major lymphoid lineages (*Chen et al., 2004; Li et al., 2007; Neilson et al., 2007*).

Immunological studies in mice have shown that miR-155 is expressed in germinal center B cells, and is required for normal germinal center formation, antibody titers in response to antigen, and plasma producing increased levels of IL-4 and less interferon gamma than controls (*Thai et al., 2007; Rodriguez et al., 2007*).

In both mouse and human cells, the earliest stages of erythroid cell differentiation are accompanied by dramatic upregulation of miR-451 (*Zhan et al., 2007; Masaki et al., 2007*). Indeed, even peripheral red blood cell preparations show significant amounts of miR-451, perhaps indicating a role for this microRNA in the final regulation of mRNA in reticulocytes (*Rathjen et al., 2006*), cell differentiation or persistence (*Thai et al., 2007; Rodriguez et al., 2007; Vigorito et al., 2007*). Normal functioning of T lymphocytes and dendritic cells also appears to depend on this microRNA (*Thai et al., 2007; Rodriguez et al., 2007*). In absence of miR-155, helper T cells deviate toward the T_H2 phenotype, producing increased levels of IL-4 and less interferon gamma than controls (*Thai et al., 2007*).

1.3.6 Nervous System

miR-9, miR-124 and miR-128 are among the microRNAs most highly and specifically expressed in the mammalian brain (*Cao et al., 2006; Lagos-Quintana et al., 2002*). Deletion of the well-conserved miR-9 in fruit flies causes duplication of sensory neurons and sensory organs in development (*Li et al 2006*). The effects of overexpression or temporarily knocking

down miR-124 in chick neural tubes, in contrast, are somewhat controversial, and show, at most, a modest impact of this microRNA on neuronal differentiation (*Cao et al., 2007; Visvanathan et al., 2007*). Neuronal differentiation in neuroblastoma and embryonal carcinoma cell lines in vitro is enhanced by miR-124, at least in part due to miR-124 inhibiting expression of regulator of alternate mRNA splicing, PTBP1 (*Makeyev et al., 2007*). Given the apparent rich diversity of microRNAs expressed at lowel levels in the mammalian CNS, subtle phenotypes or highly cell type-specific microRNA roles may be the rule in this organ (*Berezikov et al., 2006*).

1.3.7 Extracellular miRNA

While the majority of miRNAs are found intracellularly, a number of miRNAs have recently been detected outside of cells, including in various body fluids (e.g., serum, plasma, saliva, urine and milk) (*Chen et al., 2008; Lawrie et al., 2008; Mitchell et al., 2008; Park et al., 2009; Chen et al., 2010; Hanke et al., 2010*). Furthermore, alterations in the level and composition of these extracellular circulating miRNAs are tightly correlated with various health problems, including cancers (*Chen et al., 2008; Lawrie et al., 2008; Mitchell et al., 2008; Park et al., 2009; Hanke et al., 2010*), diabetes (*Chen et al., 2008*) and tissue injury (*Ji et al., 2009; Laterza et al., 2009; Wang et al., 2009*). These results firmly establish the quantification of circulating miRNAs as an extremely promising biomarker to assess and monitor the body's pathophysiological status.

Extracellular miRNAs circulate in the body fluids with a high concentration and sufficient integrity despite high extracellular ribonuclease (RNase) activity, indicating that extracellular miRNAs are likely packaged in some manner to shield them from digestion. Indeed, naked miRNAs added to plasma are immediately degraded, whereas circulating miRNAs are stable for hours under the same conditions (*Mitchell et al., 2008*). Further studies have also shown that circulating miRNAs are protected from and resistant to harsh conditions such as extreme temperatures, extreme pHs or freeze-thaw cycles (*Chen et al., 2008*). This phenomenon raises intriguing questions regarding the mechanism of circulating miRNA stability. At present, there are at least three possibilities for the remarkable stability of circulating miRNAs and their sources: (1) they are passively leaked from broken cells during tumorigenesis or tissue injury (*Chen et al., 2008; Mitchell et al., 2008*); (2) they are packaged in small membranous vesicles, including exosomes, shedding vesicles and apoptotic bodies, which accounts for the release of miRNAs into circulation and offers protection against RNase activity (*Valadi et al., 2007; Zernecke et al., 2009; Kosaka et al., 2010; Zhang et al., 2010*); (3) they are protected by the formation of a protein-miRNA complex (Fig. 3).

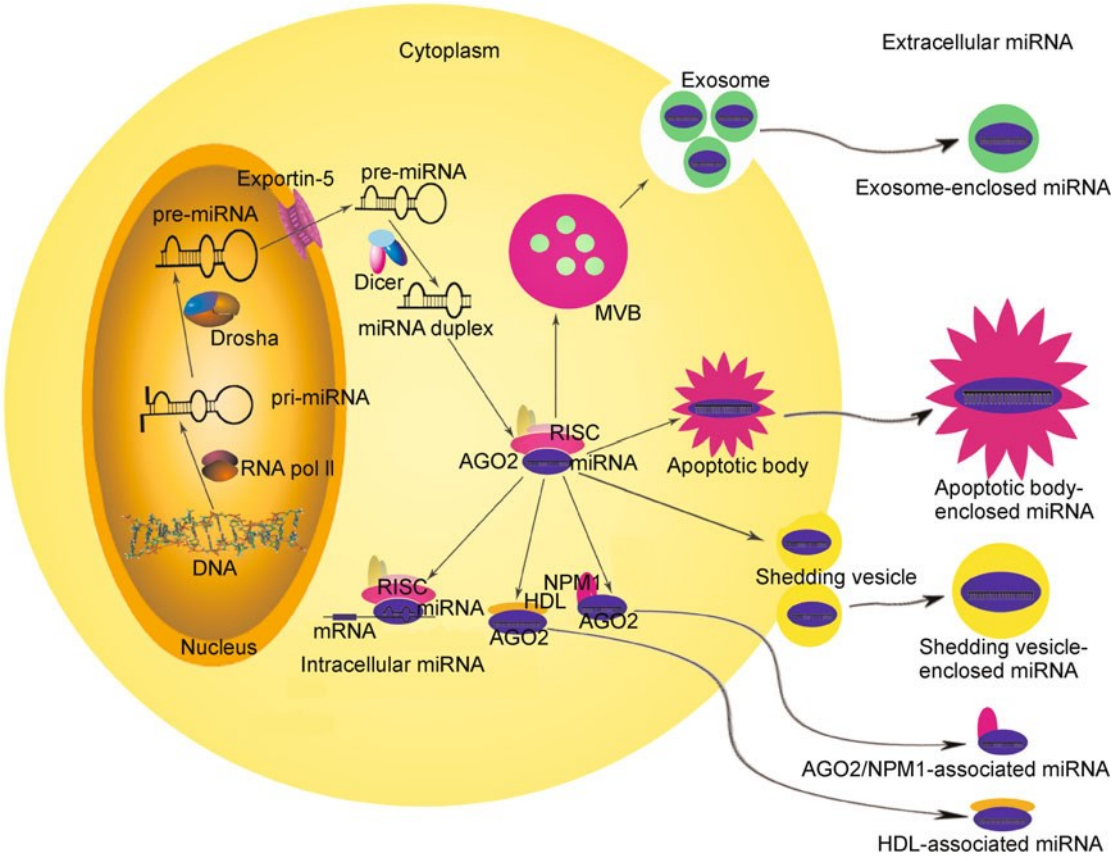


Figure 3. A model of the secretion of miRNAs. After being processed to the mature form, some miRNAs can bind to complementary sequences on target mRNAs to repress translation or trigger mRNA cleavage. Other miRNAs are packaged and transported to the extracellular environment via three different pathways: (1) they are enclosed within membranous vesicles, including exosomes, shedding vesicles and apoptotic bodies; (2) they are associated with lipoproteins, such as HDL; (3) they are associated with RNA-binding proteins, such as AGO2 and NPM1. AGO2, Argonaute 2; NPM1, nucleophosmin1.

Some studies have demonstrated that many extracellular miRNAs are detectable in conjunction with proteins or lipoproteins, but devoid of membrane vesicles, suggesting the existence of non-vesicleenclosed, RNA-binding protein-associated miRNAs in extracellular fluids, including those bound to AGO2, nucleophosmin1 (NPM1), and high-density lipoprotein (HDL) (*Wang et al., 2010; Arroyo et al., 2011; Turchinovich et al., 2011; Vickers et al., 2011*). Recent studies indicate that miRNAs released from donor cells that are either enclosed in vesicles (exosomes, shedding vesicles and apoptotic bodies) or associated with lipoproteins (HDL) are active and can function as secreted molecules to influence the recipient's cell phenotype (*Zernecke et al., 2009; Kosaka et al., 2010; Zhang et al., 2010; Vickers et al., 2011*). The difference between vesicle enclosed and lipoprotein-associated miRNAs is largely unknown. Their different secretion mechanisms suggest that they may originate from different cell types and, therefore, have different fates and functions. For example, miRNAs detected only in the lipoprotein-associated fractions may be generated by cells with lipoprotein transport pathways. By contrast, miRNAs that are predominantly packaged in

vesicles may originate from cell types known to generate abundant vesicles. However, miRNAs associated with other types of RNA-binding proteins (e.g., AGO2 and NPM1) may also be actively released from donor cells and taken up by recipient cells, although the direct evidence remains elusive.

1.4 MICRORNAs IN CANCER

1.4.1 miRNAs and tumorigenesis

Tumors result from pathological changes caused by dysregulation of cell proliferation and apoptosis. Abnormal expression of oncogenes and tumor suppressor genes has been widely accepted as the molecular mechanism of tumorigenesis. However, this traditional concept is being challenged by the discovery of non-coding RNAs. Recent studies have shown that miRNAs play important regulatory roles in tumorigenesis. miRNAs are expressed in a variety of tumors, exhibiting abnormal functions; they are involved in tumorigenesis and tumor development by negatively regulating protein-coding genes related to these processes. On one hand, downregulation or null expression of a tumor suppressor miRNA can lead to the expression of the miRNA target genes that promote tumorigenesis, causing excessive cell proliferation and abnormal differentiation and resulting in tumorigenesis (*Jun et al., 2011*).

On the other hand, overexpression of oncogenic miRNAs can lead to decreased expression of the target genes with tumor suppressor functions, thereby promoting tumorigenesis and tumor development. For example, overexpression of miR-221 or miR-222 inhibits the expression of the Kit protein, resulting in the dedifferentiation of thyrocytes and tumorigenesis (*He et al., 2005*). Deficiency of miR-15a and miR-16-1 expression can lead to overexpression of Bcl-2, an important anti-apoptotic factor, resulting in decreased apoptosis, thereby promoting tumorigenesis and tumor development. Some studies have found that miR-150 is significantly overexpressed in gastric cancer tissues and promotes gastric cancer cell proliferation through EGR2 (*Wu et al., 2010*). Moreover, the expression of miR-34 is decreased in gastric cancer. Restoring miR-34 expression in the gastric cancer cells with a mutant p53 significantly inhibits the protein expression of Bcl-2, Notch, and HMGA2, causing cell cycle arrest in G1 phase and cell growth inhibition. It also increases the activity of caspase-3 to promote apoptosis and inhibits the formation and growth of gastric cancer stem cell spheroids (*Ji et al., 2008*).

An interesting phenomenon found in recent studies of tumor miRNA expression profiles is that the miRNAs abnormally expressed in tumors usually exhibit reduced expression levels compared to their expression levels in normal tissues (*Lu et al., 2005*). This phenomenon may reflect a higher proliferation rate and a less differentiated state of tumor cells. Another explanation is that the cells

with low levels of miRNAs are selected for during tumorigenesis due to their proliferative and survival advantages. These two possibilities are not mutually exclusive. In fact, both of them are supported by experimental evidence. For instance, after differentiation induction, HL60 cells show significantly increased miRNA expression, consistent with the fact that the differentiation state of the cells is maintained by the enhancement of miRNA transcription. Moreover, studies in lung cancer models have indicated that inhibiting miRNA biogenesis by genetic approaches or RNAi can promote tumorigenesis and tumor development (*Kumar et al., 2007*). In addition, c-Myc can induce the universal silencing of miRNA transcription (*Chang et al., 2008*), providing a possible mechanism for miRNA downregulation in malignant cells. These findings suggest that the majority of miRNAs may function as tumor suppressors in tumorigenesis.

1.4.2 OncomiRs and metastamiRs

In general, a miRNA able to promote cancer targets mRNAs encoding tumor suppressor proteins, while miRNAs exhibiting tumor suppressor properties usually target mRNAs encoding oncoproteins. miRNAs which have been demonstrated to play a crucial role in the initiation and progression of human cancer are defined as oncogenic miRNAs (oncomiRs) (*Ge et al., 2013; Rather et al., 2013; Shu et al., 2011; Haug et al., 2011; Tang et al., 2011; Ma et al., 2010; Mercatelli et al., 2008*). Moreover, miRNAs have been firmly demonstrated to be involved in cancer metastasis (metastamiRs) (*Hurst et al., 2009; Wotschofsky et al., 2012; Taylor et al., 2013; Welch et al., 2013*). Thus, therapeutic strategies involving miRNA silencing have been suggested, based on the roles of these small non-coding RNAs as oncogenes (*Ge et al., 2013; Rather et al., 2013; Shu et al., 2011; Haug et al., 2011; Tang et al., 2011; Ma et al., 2010; Mercatelli et al., 2008*).

Another very interesting feature of miRNAs has been found by studying cancer-associated miRNAs in different experimental model systems; cancer-specific miRNAs are present in extracellular body fluids and may play a crucial role in the cross-talk between cancer cells and surrounding normal cells (*Moldovan et al., 2013; Chen et al., 2012; Kosaka et al., 2011; Chen et al 2012; Ramachandran et al., 2012; Muralidharan et al, 2010*). Of note, evidence of the presence of miRNAs in serum, plasma and saliva supports their potential as an additional set of biomarkers for cancer. As previously mentioned, extracellular miRNAs are protected, for example, by exosome-like structures, small intraluminal vesicles shed from a variety of cells (including cancer cells), with a biogenesis connected with the endosomal sorting complex required for transport machinery in multivesicular bodies. These extracellular structures, originally considered as a ‘garbage bag’ devoted to discarding degraded proteins, are now considered to play an important role as an intercellular communication tool. It is still unclear as to whether these exosome-associated miRNAs

occur as a result of tumor cell death and lyses, or are actively excreted from tumor cells into the microenvironment. However, this novel secretory machinery of miRNAs may be involved in tumor-associated features, such as the enhancement of angiogenesis, the increase of cytokine secretion and migration to pre-metastatic niche. Table 1 illustrates a summarized list of oncomiRs and metastamiRs.

Cells/Tissues	***miRNA target***	***Modulated mRNA***	***Effects following antagomiR treatement***	***Authors***
Human glioblastoma	miR-27a	FOXO3a	Suppression of U87 growth in vitro and in vivo	Ge et al.
Cutaneous squamous cell carcinoma (SCC)	miR-155	CDC73	Decreased cell viability, increased apoptosis and marked regression of xenografts in nude mice	Rather et al.
Malignant astrocytoma cells	miR-335	Daam1	Growth arrest, cell apoptosis, invasion repression and marked regression of astrocytoma xenografts	Shu et al.
Neuroblastoma	miR-92	DKK3	Increased released of the tumor suppressor Dickkopf-3 (DKK3), a secreted protein of the DKK family of Wnt regulators	Haug et al.
Glioma	miR-381	LRRC4	Decreased cell proliferation and tumor growth	Tang et al.
Breast cancer	miR-10b	Hoxd10	Suppression of formation of lung	Ma et al.

			metastases	
Prostate cancer	miR-221/miR-222	p27	Reduction of tumor growth	Mercatelli et al.

Table 1: Exaples of oncomiRs suitable for antagomiR-based miRNA targeted therapy of cancer.

1.4.3 Oncosuppressor microRNAs

In addition to oncogenic activities, miRNAs exhibit, oncosuppressor properties by targeting mRNAs encoding oncoproteins (*Scheibner et al 2012; Endo et al., 2013; Liang et al., 2013; Thomas et al., 2012; Ibrahim et al., 2011; Wiggins et al., 2010; Trang et al., 2011; Wu et al., 2013; Huang et al., 2013*). *Piovan et al* recently explored the interaction between certain miRNAs and transcriptional factors involved in determining cell fate, including the well known 'genome guardian', p53 (*Piovan et al., 2012*). They demonstrated that miR-205, an oncosuppressive miRNA lost in breast cancer, is directly transactivated by the oncosuppressor p53. Moreover, evaluating miR-205 expression in a panel of cell lines belonging to the highly aggressive triple-negative (estrogen receptor (ER), progesterone receptor (PR) and Her2/neu) breast cancer subtype, which still lacks an effective targeted therapy and is characterized by an extremely undifferentiated mesenchymal phenotype, the authors demonstrated that this miRNA is critically downregulated compared with a normal cell line. The re-expression of miR-205 strongly reduced cell proliferation, cell cycle progression and clonogenic potential *in vitro*, and inhibited tumor growth *in vivo*. The tumor suppressor activity of miR-205 is partially exerted by targeting E2F1, one of the master regulators of cell cycle progression, and LAMC1, a component of the extracellular matrix involved in cell adhesion, proliferation and migration. In another study, Lee et al. (*Lee et al., 2011*), demonstrated that an estrogen-downregulated miRNA, miR-34b, acts as an oncosuppressor that targets cyclin D1 and Jagged-1 (JAG1) in an ERα-positive/wild-type p53 breast cancer cell line (MCF-7), as well as in ovarian and endometrial cells, but not in ERα-negative or mutant p53 breast cancer cell lines (T47D, MBA-MB-361 and MDA-MB-435). The negative association between ERα and miR-34b expression levels has also been found in ERα-positive breast cancer patients. In addition, the overexpression of miR-34b has been shown to inhibit ERα-positive breast tumor growth in an orthotopic mammary fat pad xenograft mouse model. Table 2 illustrates a summarized list of oncosuppressor miRNAs (*Iorio et al., 2012; Xu et al., 2013; He et al., 2013; Iorio et al., 2009*).

Tumor type	***miRNA***	***Modulated mRNA***	***Effects following pre-miRNA administration***	***Authors***
Acute leukemia	miR-27a	Bax and Bad	Inibition of cell growth due, at least in part, to increased cellular apoptosis	Scheibner et al.
Oral squamous cell carcinoma (OSCC)	miR-596	LGAL3BP	Growth inhibition	Endo et al.
Breast cancer	miR-302	AKT1 and RAD52	Sensitized radioresistant breast cancer cells to ionizing radiation	Liang et al.
Chronic myelogenous leukemia (CML) cells	miR-33a	Pim-1	Decelerated cell proliferation	Thomas et al.
Colon carcinoma	miR-33a	Pim-1	Reduced tumor proliferation	Ibrahim et al.
Colon carcinoma	miR-145	c-Myc and ERK5	Reduced tumor proliferation and increased apoptosis	Ibrahim et al.
Lung cancer	miR-34a	Repression of c-Met, Bcl-2; partial repression of CDK4	Block of tumor growth	Wiggins et al.
Lung cancer	miR-let7	Negative regulation of the cell cycle oncogenes RAS, MYC and HMGA2	Reduction of tumor growth	Trang et al.
Non-small cell lung adenocarcinomas,	miR-29b	CDK6, DNMT3B,	Inibhition of tumorigenicity *in*	Wu et al.

A549 cells		MCL-1	*vivo*	
Acute myeloid leukemia (AML)	miR-29b	Downregulation of DNMTs, CDK6, SP1, KIT and FLT3	Decreased AML cell growth and impairement of colony formation; longer survival of treated mice; improvement of anti-leukemic activity of decitabine	Huang et al.

Table 2: miRNAs acting as tumor suppressor genes and are suitable for replecement theraphy of cancer.

1.4.4 Role of secreted miRNAs in tumor progression

The tumor microenvironment plays a critical role in cancer progression. Cancer cells can influence normal cells to abandon their homeostatic activities and instead support the neoplastic nature of the tumor. The dynamic crosstalk between cancer cells and normal cells in the microenvironment is crucial to the progression of disease. Crosstalk can occur through secreted molecules and paracrine signalling (*Muralidharan-Chari et al., 2010*). The secreted molecules are no longer limited to cytokines, chemokines, growth factors or other protein molecules but now also include secreted miRNAs. *Skog et al.* reported that particular mRNAs and miRNAs are highly enriched in microvesicles from primary human glioblastoma cells (*Skog et al., 2008*). Glioblastoma-derived RNA contained in microvesicles is functional and is taken up by and processed in the human brain microvascular endothelial cell line HBMVEC, generating a functional protein (*Skog et al., 2008*). These results suggest that the tumor-derived microvesicles can modify the surrounding normal cells by changing their translational profile. In addition, the tumor-specific mRNA and miRNAs characteristic of gliomas, such as EGFRvIII and miR-21, could be detected in serum microvesicles of glioblastoma patients (*Skog et al., 2008*).

This finding led to the hypothesis that tumor cells use exosomes to transport genetic information, including miRNAs, to surrounding cells, thereby supporting tumor growth and progression. Furthermore, *Ohshima et al.* revealed that the let-7 miRNA family was enriched in the extracellular exosomes from a metastatic gastric cancer cell line AZ-P7a, while low metastatic AZ-521, as well as other cancer cell lines, showed no such enrichment (*Ohshima et al., 2010*). Because let-7 miRNAs generally function as tumor suppressors that target oncogenes such as RAS and high mobility group A2 (HMGA2), they proposed that cancer cells selectively secrete let-7 miRNAs into

the extracellular environment via exosomes, reducing the anti-tumorigenic effect within the cells and facilitating oncogenesis and metastasis (*Ohshima et al., 2010*).

Therefore, miRNAs can not only promote the development of primary tumors, but also affect tumor progression, including tumor metastasis (*Ma and Weinberg, 2008; Dumont and Tlsty, 2009; Nicoloso et al., 2009; Zhang et al., 2009; Baranwal and Alahari, 2010; Ding et al., 2010; Khew-Goodall and Goodall, 2010; Li et al., 2010; Ma et al., 2010; Sachdeva and Mo, 2010; Santarpia et al., 2010; Schmittgen, 2010; Tian et al., 2010; Zhang et al., 2010*). Both mutation (*Gardner and Vinther, 2008*) and misexpression (*Santarpia et al., 2010; Zhang et al., 2010*) of miRNAs can affect their normal functions, leading to abnormal expression of their target genes. Thus, tumor metastasis may be affected when the target genes are related to tumor cell migration, invasion, resistance, and other metastatic phenotypes. Among several known miRNAs promoting tumor metastasis, miR-10b and miR-373 are particularly prominent. miR-10b is a direct target gene of the transcription factor Twist1 that promotes epithelial-mesenchymal transition and tumor metastasis. The expression of miR-10b is markedly upregulated in human breast cancer cells of high metastatic potential; the invasiveness of these cells decreases by 10-fold if the activity of miR-10b is blocked by an antisense oligonucleotide. Overexpression of miR-10b in breast cancer cells with low metastatic potential leads to a significant increase in the invasiveness of the tumor cells. Six weeks after orthotopic inoculation of highly miR-10b-expressing breast cancer cells into the breast of immunodeficient young female mice, tumors were found at the inoculation sites in all of the inoculated mice, together with apparent interstitial and vascular infiltration; distant metastasis appeared in all of these mice 11 weeks after the inoculation. No invasion or metastasis was observed when using low miR-10b expressing breast cancer cells. miR-10b promotes the invasion and metastasis of breast cancer cells through inhibiting its target gene HOXD10 to increase the expression of RhoC (Ma et al., 2007). miR-335 can induce cell morphological changes to reduce the invasiveness and metastasis of breast cancer cells through directly regulating its targets, the progenitor cell-regulating transcription factor SOX4 and the extracellular matrix component cadherin C (*Tavazoie et al., 2008*). miR-29c can inhibit the metastasis of nasopharyngeal carcinoma by regulating a variety of extracellular matrix proteins, including inhibiting collagen and laminin γ1 (*Sengupta et al., 2008*). miR- 373 was identified from in vitro screening for enhanced tumor cell migration phenotypes (*Huang et al., 2008*), and its role in promoting metastasis has been confirmed *in vivo*. It should be noted that miR-373 also exhibits the characteristics of an oncogene during the tumorigenesis of testicular germ cell tumors (*Voorhoeve et al., 2006*). It has been suggested that its oncogenic characteristics of promoting metastasis are a result of regulating different target genes.

1.4.5 miRNAs and cancer drug resistance

In addition to the findings that miRNAs are associated with tumor pathogenesis and development, miRNAs are closely related to the drug resistance of tumor cells (*Lwin et al., 2010; Ma et al., 2010; Zheng et al., 2010; Akao et al., 2011*). miRNAs affect the sensitivity of tumor cells to cytotoxic drugs (*Xia et al., 2008*), biologically targeted drugs (*Miller et al., 2008; Weiss et al., 2008*), endocrine drugs (*Garofalo et al., 2008; Zhao et al., 2008*), and cytokine drugs (*Kovalchuk et al., 2008*). For example, Kovalchuk et al. (2008) found that the expression of miR-451 is significantly reduced in the doxorubicin-resistant breast cancer cell line MCF27/DOX and that restoring miR-451 expression by transfection can increase the sensitivity of MCF27/DOX cells to doxorubicin. Si et al. (2007) discovered that a miR-21 inhibitor can significantly increase the sensitivity of breast cancer cells to topotecan. The studies by Venturini et al. (2007) showed that miR-17-19b transfection can remarkably increase the imatinib induced apoptosis of the acute myeloid leukemia cells K562.

miRNAs appear to play important roles in tumor drug resistance by regulating the expression of drug resistance proteins, and targeted intervention of miRNAs may effectively reverse tumor drug resistance (Table 3).

Cancer type	***miRNA***	***Target gene***	***Reference***
Breast cancer	miR-451 miR221/222 miR-200 family miR-328	Mdr1/Pglycoprotein P27(Kip1) E-cadherin BCRP/ABCG2	Kovalchuk et al. Miller et al. Tryndyak et al Pan et al.
Gastric cancer	miR-15a miR-16	BCl-2	Xia et al.
Non-small cell lung cancer	miR221/222	Kit, p27	Zhao et al.
Ovarian cancer	miR-214 miR-130a miR-27a miR-451	PTEN M-CSF Mdr1/p-glycoprotein	Zhu et al.
Prostate cancer	miR-34a miR-148a	Sirt-1 MSK1	Fujita et al.
Colon cancer	miR-519c	ABCG2	Ibrahim et al.

	miR-34a	Sirt1/E2F3	
Cervix carcinoma	miR-27a miR-451	Mdr1/p-glycoprotein	Zhu et al.
Esophageal carcinoma	miR-let7	Mdr1/p-glycoprotein Bcl-2	Zhang et al.
B-cell lymphomas	miR-181a	Bim	Lwin et al.

Table 3: Reported studies on miRNAs and tumor drug resistance.

1.4.6 miRNAs and tumor diagnosis

miRNAs can be used to identify benign and malignant lesions and to determine the prognosis of cancer patients. High-throughput miRNA detection technologies have been emerging in recent years, including miRNA chips, miRNA expression profiling using magnetic bead–based flow cytometry, and miRNA qPCR assays. These methods have been used to identify a variety of tumor-specific miRNAs through parallel comparison between cancer tissues and the adjacent tissues. miRNAs are differentially expressed between tumor cells and normal cells, as well as among the tumor cells originating from different tissues. The discovery of miRNA expression features can help not only distinguish between benign and malignant lesions, but also determine the degree of tumor malignancy and prognosis, thereby providing the basis for personalized therapy (*Alvarez-Garcia and Miska, 2005; Calin et al., 2005*). For example, Calin et al. (2005) selected 13 out of 190 miRNAs as a set of miRNA expression markers for chronic lymphocytic leukemia; this miRNA set can not only distinguish between malignant and normal B lymphocytes, but also identify the chronic lymphocytic leukemia cases with high expression of 70-kD zeta-associated protein (ZAP70) or a mutated IgV (H) gene, which are associated with high malignancy, strong invasiveness, and poor prognosis. In non-small cell lung cancer, miR-155 upregulation and let-7 downregulation indicate poor prognosis. In colon cancer patients, high expression of miR-21 indicates poor prognosis. In other studies, it has been found that a characteristic spectrum composed of seven miRNAs (miR-10b, miR-21, miR- 223, miR-338, let-7a, miR-30a-5p, miR-126) is sufficient to predict the survival of gastric cancer patients (*Li et al., 2010*). Because miRNAs are relatively stable, it becomes more and more promising to use miRNAs as new tumor markers.

1.4.7 miRNAs can distinguish different malignant phenotypes of the same tumor

Different malignant phenotypes of tumors originating from the same tissue can result in markedly different therapeutic outcomes and prognosis. miRNAs can distinguish not only normal cells from tumor cells, but also the different malignant phenotypes of tumor cells originating from the same tissue. For example, *Budhu et al.* (*2008*) examined the miRNA expression profiles in cancer tissues and the corresponding adjacent tissues from 241 hepatic cancer cases using miRNA microarray. These cancer specimens included both invasive and non-invasive samples. Their results showed that the levels of 20 miRNAs can accurately predict hepatic cancer metastasis, which are closely related to post-operative relapse and the survival of hepatic cancer patients. Compared to their expression in drug-sensitive gastric cancer cells, miR-15a and miR-16b were downregulated in drug-resistant gastric cancer cells (*Xia et al., 2008*), and miR-218 was downregulated in the gastric cancer cells with high metastatic potential (*Tie et al., 2010*).

1.4.8 miRNAs reflect the origin of tumor tissues

By examining the samples from 540 cases, including 363 cases of six types of malignancies with high incidence rates and 177 normal controls, *Volinia et al.* (*2006*) discovered that specific miRNA expression profiles can not only distinguish normal cells from tumor cells, but also reflect the origin of tumor tissues. *Lu et al.* (*2005*) analyzed the miRNAs in 334 samples from 217 mammalian species and found that tumors originating from the organs that develop from embryonic endoderm, such as stomach, intestine, and liver, displayed similar miRNA expression patterns, whereas the miRNA expression patterns of leukemia were significantly different from those of solid tumors, suggesting that characteristic miRNA expression profiles possess diagnostic value in the identification of tumor tissue origin and exhibit great potential in the diagnosis of metastatic tumors with unknown primary tumor origin. miRNA expression profiles can also accurately reflect the progression and differentiation state of tumor tissues, especially for the poorly differentiated malignant tumors. Compared with the previously reported mRNA expression profiles, miRNA expression profiles provide a more accurate reflection of tumor status (*Alvarez- Garcia and Miska, 2005*).

1.4.9 Circulating miRNAs, new tumor markers

The discovery of circulating miRNAs further promoted investigations of miRNAs as biological markers in tumor diagnosis and prognosis. *Lawrie et al* (*2008*) first observed high expression of miR-21 in sera from patients with diffuse large B cell lymphoma and the close correlation of miR-21 expression with disease recurrence and patient survival. *Mitchell et al.* (*2008*) isolated RNAs of 18-24 nucleotides from the plasma of healthy volunteers and constructed a small RNA library. They

found 91 known and 4 unknown miRNAs by sequencing analysis. Subsequently, 25 patients with metastatic prostate cancer and 25 healthy volunteers were separated into different groups, whose sera were examined. They found that the plasma miR-141 levels in prostate cancer patients were significantly higher than those in the normal control group. Thus, prostate cancer patients can be effectively distinguished from healthy individuals based on their miR-141 expression levels, indicating that miR-141 can be used as a circulating miRNA marker for the detection of prostate cancer. *Chen et al.* (*2008*) analyzed the miRNA expression in the sera from patients with lung cancer, colon cancer, or diabetes using Solexa sequencing and quantitative PCR.

The results showed that these diseases all have their characteristic serum miRNA expression patterns. For example, 63 miRNAs were expressed in the sera of patients with lung cancer, but not in the sera of healthy individuals. Ten miRNAs were common to the sera of lung cancer and colon cancer patients. *Resnick et al.* (*2009*) examined the miRNA expression profile in plasma from eight ovarian cancer patients and found 23 differentially expressed miRNAs, among which 10 molecules had been previously reported in solid ovarian tumors. Verification in plasma from 19 ovarian cancer patients by qRT-PCR showed that miR-21, miR-29a, miR-92, miR-93, and miR-126 were remarkably upregulated in these patients, whereas miR-155, miR-127, and miR-99b were significantly downregulated.

Nevertheless, that study suggests that specific plasma miRNAs may serve as molecule markers that are detectable earlier than the traditional tumor markers used in tumor diagnosis and in efficacy assessment. These studies demonstrated that miRNAs are broadly present in the sera of healthy individuals and of patients with different diseases, including cancer. The types and quantities of miRNAs present in serum and plasma vary with physiological and pathological conditions. Specific miRNA expression profiles in the sera of tumor patients provide a new method for early diagnosis, classification, prognosis, efficacy assessment, and relapse monitoring of tumors, bringing new hope to noninvasive tumor diagnosis (*Tie et al., 2011*).

1.4.10 miRNAs related cancer diagnosis, polymorphisms and AMO

As already mentioned, the important roles of miRNAs in cancer and their potential applications as useful and effective targets have generated great interest in cancer gene therapy strategies, as well as diagnosis, classification, prognosis and risk factor evaluations. Based on microarrays for miRNA expression profiling studies, differences in miRNA expression could be detected between normal and cancer tissues, which can classify different tumor types and tumor grades (*Visone et al., 2007; Yanaihara et al., 2006; Trang et al., 2008*). Certain miRNA signatures are correlated with prognosis and can potentially be used to determine the specific course of treatment. *Michael et al. (2003)* found aberrant miRNA expression in solid tumors as they identified 28 different miRNAs in

colonic adenocarcinoma compared with normal mucosa. miR-143 and -145 were significantly down-regulated in the cancer. Similar situation was detected in other cancers, as miR-221, -222, and -146 in papillary thyroid carcinoma (*He et al., 2005*), miR-21 and -155 in pancreatic cancer (*Bloomston et al., 2007*), and miR-141 in prostate cancer (*Mitchell et al., 2008*). Through analyzing the expression of 217 miRNAs in 334 samples that included primary tumors, tumor-derived cell lines and normal tissues, *Lu et al. (2005)* found that miRNA profiles can distinguish between normal and cancer tissues, separate different cancer types, stratify the cancer differentiation state and cluster sample groups according to their embryonic lineage.

Single nucleotide polymorphisms (SNPs) within the miRNA coding genes or within miRNA target genes are likely to be deleterious and can affect an individual‘s risk to develop diseases such as cancers. *Yu et al. (2007)* found that 12 miRNA-related SNPs showed an aberrant allele frequency in human cancers. *Chin et al. (2008)* identified an SNP in let-7 complementary site 6 (LCS6) in the KRAS 3’ UTR that is associated with smoking-induced lung cancer risk. This variant allele is found in 20% of the 74 non-small cell lung carcinoma patients in the study. These unique miRNA expression signatures might be the hallmarks of tumor progressions and prognosis evaluations.

Many miRNAs, have great potential in tumorigenesis, tumor invasion, metastasis, malignant progression, and poor prognosis. In *in vitro* and *in vivo* experiments, it has been confirmed that knockdown of certain miRNAs could change the tumor progression and biological characteristics as potential therapeutic targets (*Bonci et al., 2008*). In cell culture and xenograft mice models, synthetic anti-miRNA oligonucleotides (AMOs) with 2‘-O-methyl modification have been shown to effectively inhibit endogenous miRNAs. *Krützfeldt et al. (2005)* studied the utility of AMOs in vivo through intravenous injection of modified AMOs to target the liver-specific miR-122. Impressively, a single injection of 240 $mg \cdot kg^{-1}$ body weight conferred specific miR-122 silencing for up to 23 days. As an alternative to 2′-hydroxyl-modified AMOs, lock nucleic acid based oligonucleotides (LNA-antimiR) have been shown to be more stable and less toxic in inhibiting endogenous miRNAs in vivo. *Kota et al. (2099)* showed that the systemic delivery of a single miRNA could cause tumor regression in a mice model of liver cancer. They delivered adeno-associated virus 8 (AAV8)-expressing miR-26a intravenously in Myc-induced mice harboring preformed liver tumors. After 3 weeks, they observed a significant regression of tumors in mice with the miR-26a treatment. These findings indicate a possibility of specific miRNAs-target therapy.

1.4.11 miRNAs and tumor therapy

With the continuing discovery of the roles of miRNAs in tumorigenesis and tumor development, people have started to explore the use of miRNAs in tumor treatment. A single miRNA can simultaneously regulate multiple protein-coding genes and a number of signaling pathways associated with tumor growth and proliferation, metastasis, drug resistance, and other malignant phenotypes (Fig. 4).

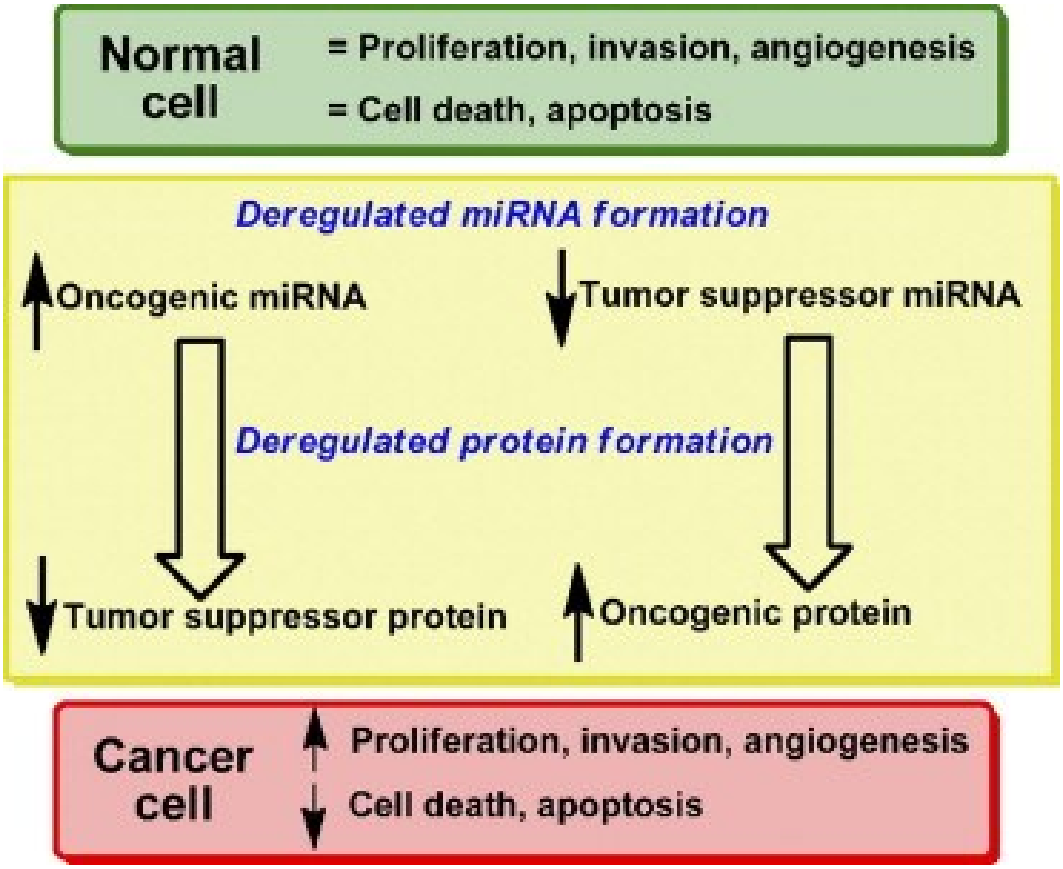


Figure 4: Deregulated microRNA biogenesis and tumorigenesis. Both reduced expression of miRNA acting as tumor suppressor and increased expression of miRNA acting as oncogenic miRNA can alter the synthesis of either oncogenic protein or tumor suppressor protein and lead to tumor formation.

Therefore, miRNAs exhibit advantages over individual protein-coding genes for treating tumors that involve alterations in multiple genes. For example, miR-31 can simultaneously regulate five proteins involved in tumor metastasis, RhoA, RDX, MMP16, Fzd3, and ITGA5, in breast cancer cells. It inhibits multiple steps in metastasis, including tumor cell migration, invasion, and colony formation in distant organs. Exogenous overexpression of miR-31 can significantly suppress the metastasis of breast cancer cells both in vitro and in vivo (*Valastyan et al., 2009, 2010; Valastyan and Weinberg, 2010*). In head and neck tumors, miR-204 can simultaneously regulate more than 60 target proteins involved in multiple tumor-associated signaling pathways (*Lee et al., 2010*), suggesting that miR-204 is a promising target for the treatment of head and neck tumors.

As already mentioned, tumor-associated miRNAs can be divided into two categories: tumor-promoting miRNAs and tumor-suppressing miRNAs. Tumor-promoting miRNAs are highly expressed in tumors. A number of methods can be used to downregulate or to suppress the expression of tumor-promoting miRNAs, including antagomirs, antimiRNA oligonucleotides and miRNA sponges. Tumor suppressing miRNAs are not expressed or are expressed at low levels in tumors. Thus, tumor treatment can be exerted by introducing corresponding exogenous miRNAs. The fact that a single miRNA can regulate multiple target genes provides new approaches for gene therapy based on RNAi. Because tumorigenesis and tumor progression are often regulated by multiple genes, an artificial miRNA can be designed to target multiple genes that are highly expressed in tumors, thereby simultaneously suppressing the expression of multiple oncoproteins. This effect cannot be achieved by traditional RNAi technology. Moreover, miRNA genes are often distributed in clusters. For example, the pri-miRNA of the miR-17-92 cluster contains seven independent mature miRNAs. Therefore, we can simulate the structure of the miR-17-92 precursor, design a structure for the expression of multiple miRNAs from a single promoter, and induce the interference of multiple oncogenes (*Tie et al., 2011*).

Altered expression of miRNAs is apparent in virtually all tumor types and includes blood-borne malignancies as well as solid tumors. The functional consequence of miRNA deregulation became evident as the introduction or repression of a single miRNA can effectively contribute to tumorigenesis or tumor progression. Numerous functional studies using cultured cancer cells and mouse models of cancer have identified miRNAs that function as conventional tumor suppressors or oncogenes. Examples of miRNAs with oncogenic activity are miR-155 and miR-17–92; in contrast, miR-15a, miR-16, as well as miRNAs of the miR-34 and let-7 families, are tumor-suppressor miRNAs (*Calin et al., 2006;Johnson et al., 2007; Johnson et al., 2005; Kumar et al., 2008; Esquela-Kersher et al., 2008; Trang et al., 2009; He et al., 2007*). The tumor-suppressive or oncogenic activity for many of these miRNAs is not limited to a particular tumor type, in agreement with the supposition that conventional cancer genes function as such regardless of tissue origin. The deregulation of some of these miRNAs also correlates with tumor differentiation status, disease stage, and patient outcome, further suggesting that aberrant miRNA function has a direct impact on tumor development. For instance, low let-7 levels and high miR-155 levels are indicative of poor survival of patients with non–small cell lung cancer (*Yanaihara et al., 2006*). Other miRNAs have specifically been implicated in early tumorigenesis or metastasis, representing unique opportunities for therapeutic intervention that will depend on the context and requirement of therapy.

The therapeutic application of miRNAs involves two strategies. One strategy is directed toward a gain of function and aims to inhibit oncogenic miRNAs by using miRNA antagonists, such as anti-

miRs, locked-nucleic acids (LNA), or antagomiRs. These miRNA antagonists are oligonucleotides with sequences complementary to the endogenous miRNA. They carry chemical modifications that enhance the affinity for the target miRNA and trap the endogenous miRNA in a configuration that is unable to be processed by RISC, or alternatively, leads to degradation of the endogenous miRNA. Small molecule inhibitors specific for certain miRNAs are also being developed to inhibit miRNA function. The second strategy, miRNA replacement, involves the reintroduction of a tumor-suppressor miRNA mimic to restore a loss of function. Although the inhibitory approach is more commonly accepted and conceptually follows rules that also apply to small molecule inhibitors and short interfering RNAs (siRNA), miRNA replacement represents a new opportunity to explore the therapeutic potential of tumor suppressors (*Johnson et al., 2007;Esquela-Kersher et al., 2008; Trang et al., 2009; Kota et al., 2009*). Therapeutically restoring the levels of tumor suppressors in tumor tissues has been investigated in the past by gene therapy; however, a practical application of this approach is still pending. Because the definition of tumor suppressors was restricted to protein-encoding genes, gene therapy usually involves the delivery of a relatively large DNA plasmid or viral vector that encodes the desired protein. Often, vector size, inefficient delivery to target tissues, and the requirement for nuclear localization represent technical challenges that limit this approach to local, rather than systemic, administration (*McCormick et al., 2001; Roth 2006*). Thus, despite a strong scientific rationale for cancer treatment, logistic obstacles associated with gene therapy leave the full therapeutic benefit of using tumor suppressors unanswered. miRNAs provide a new opportunity because, unlike proteins, miRNA mimics are substantially smaller, will merely have to enter the cytoplasm of target cells to be active, and can be delivered systemically using modes and technologies that are also used for siRNAs. Therefore, the delivery hurdle for miRNA mimics seems to be less an impediment than it is for protein encoding DNA. In addition, several other key observations support the concept of miRNA replacement therapy:

- the majority of differentially expressed miRNAs is suppressed in tumor tissue relative to normal tissues, indicating that the probability for miRNAs as tumor suppressors is greater than the probability as oncogenes (*Lu et al., 2005*);
- inhibition of endogenous miRNA processing induces oncogenic transformation and augments tumorigenesis, suggesting that the tumor suppressive role of miRNAs prevails over an oncogenic role (*Kumar et al., 2007*).

Another advantage of miRNA mimics is the fact that a miRNA mimic has the same sequence as the depleted, naturally occurring miRNA and, therefore, is expected to target the same set of mRNAs that is also regulated by the natural miRNA. Nonspecific off-target effects are unlikely as miRNA mimics are expected to behave like the natural counterpart for which the proper miRNA-mRNA

interactions have evolved over a billion years. The strongest rationale for exploring the therapeutic potential of miRNAs, however, is based on the observation that a single miRNA can regulate multiple oncogenes and oncogenic pathways that are commonly deregulated in cancer (*Esquela-Kersher et al., 2006*). Therefore, miRNAs act in accordance with our current understanding of cancer as a "pathway disease" that presumably can only be successfully treated when intervening with multiple oncogenic pathways (*Check, 2008*). The inhibitory effects induced by miRNAs on any particular target may be mild and may merely lead to a subtle reduction of protein expression; however, the simultaneous down-regulation of a broad set of targets has far-reaching biological consequences that determine the course of the cellular phenotype. The rapid and coordinated manipulation of protein levels across multiple pathways endows these regulatory RNAs with the ability to instantly switch between cellular programs. By restoring the expression of tumor suppressive miRNAs, miRNA replacement therapy seeks to reinstate those cellular programs that are active in normal cells and interfere with oncogenic programs necessary for the malignant phenotype (*Bader et al., 2010*).

To date, few tumor suppressor miRNAs have been discovered for which the proof of concept of miRNA replacement therapy has been shown in preclinical animal models of cancer (*Bader et al., 2010*).

Therapeutic miRNA mimics may be better tolerated by normal cells than cancer cells because:

- pathways activated or repressed by the miRNA mimic are already activated or repressed by the endogenous miRNA;
- administration of therapeutic miRNA mimics is only an insignificant incremental increase of what is already present in normal cells;
- normal cells are not addicted to oncogenic pathways and manage to recover from the therapeutic dose used;
- normal cells have the ability to regulate the activity or presence of the miRNA mimic, whereas cancer cells do not.

As therapeutic programs advance miRNAs closer to the clinic, it will become critical to study miRNA-induced effects in normal cells and to assess potential toxicity in higher species. Taken together, miRNA replacement has emerged as a highly promising therapeutic strategy. It encompasses several conceptual aspects of traditional gene therapy and technical features of siRNA therapeutics. However, given the fundamental differences in the approach, mechanism of action, and outcome, miRNA mimics should be viewed as a new class of therapeutics. Available data, showing that miRNAs can function as bona fide tumor suppressors and that synthetic versions of these miRNAs robustly interfere with tumor growth in animal models, strongly support the

development of miRNA mimics. In addition, recent data implicating miRNAs in self-renewing tumor-initiating cancer cells (cancer stem cells) may significantly broaden the scope of miRNA mimics and may suggest that miRNAs can become valuable tools to eliminate cancer cells frequently associated with chemoresistance, metastasis, and recurrence (*Ji et al., 2009*, *Yu et al., 2007*). The main challenge for successful translation into the clinic remains in vivo delivery, which will be the focus of future therapeutic development efforts to harness the full potential of miRNAs.

1.5 RENAL CELL CANCER

Renal cell cancer (RCC) is a relatively uncommon solid tumor, accounting for about 2% of all adult malignancies, but this rate of incidence is rising in Europe with the concomitant decrease of mortality rates (*IARC*). Currently, it is well accepted that RCC does not constitute a single biological entity but, rather, a varied group of malignancies.

1.5.1 Malignant renal cell tumors

Renal cell carcinoma

Conventional cell renal carcinoma is the most frequently described histotype of RCC and accounts for 60% to 70% of cases. Sometimes, in the absence of clear cells, diagnosis of the tumor relies on the detection of a characteristic vascular network. In most cases, clear cytoplasm cells clustered in small groups are identified even in tumors composed predominantly of cells with an eosinophilic cytoplasm (Fig. 5).

The term “conventional” is used to avoid the word clear, because not all types in this group present clear cells. At present, a peculiar set of chromosome alterations is known only for its conventional and papillary histotypes (accounting for about 85% to 90% of renal cancers), while these data are not yet available for the chromophobe nor for the collecting duct histotypes. The clear cell carcinoma type is associated mostly with germ line mutations of the von Hippel-Lindau suppressor gene, and some somatic mutations. Some of these sporadic tumors will also have a mutation in 3p, but basically these are the most common tumors to be recognized. Thus, approximately half of the cases of clear cell RCC show mutations of the VHL gene or hypermethylation of its promoter region. VHL-mutated RCC cases show a more favorable prognosis than those without VHL alterations (*Mancini et al., 2008*). Mutation of the VHL gene at 3p25–26, gene rearrangement, or promoter region hypermethylation is frequently associated with clear cell RCC (*Polascik et al., 2002*). Additional regions on 3p are associated with clear cell carcinogenesis, and loss of heterozygosity (LOH) studies have shown mutations at 3p14.2 and 3p21 to be early events in neoplastic transformation (*Velickovic et al., 1999*).

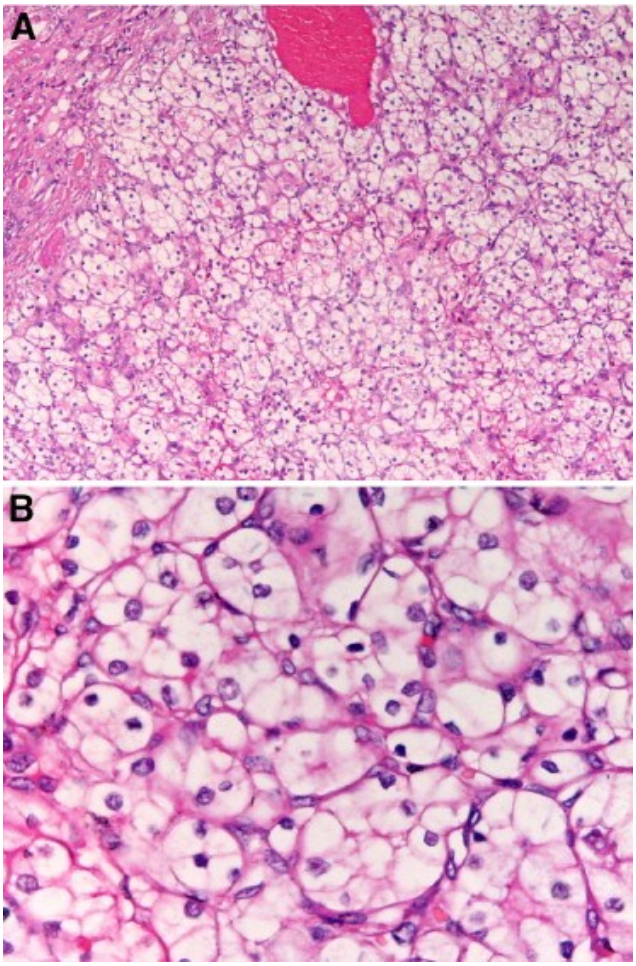


Figure 5: Malignant tumors. "Conventional" clear cell renal carcinoma, 100X(A) and 400X(B), hematoxylin and eosin.

Recent studies have demonstrated that while a variety of chromosomal abnormalities are associated with advanced tumors, and LOH at 9p13, 14q and 10q (PTEN/MMAC1) are associated with poor prognosis, gains of 5q31 correlate with a more favorable outcome (*Gunawan et al., 2001*). A recent report suggests that VHL status may have prognostic significance for patients with sporadic clear cell RCC. VHL gene, a key player in hypoxia-signaling pathway, is mutated or hypermethylated in 40% to 70% of sporadic clear RCC; it was detected in 108 of 187 RCC tumor samples, and VHL alterations were strongly associated with better cancer-free survival for 134 patients with stage I–III clear cell RCC treated by radical nephrectomy. When VHL suppressor protein function is lost or in hypoxia, the hypoxia-inducible factor (HIPα) is accumulating at intracellular level and transactivates many genes responsible for the ability of the cancers to adapt to a hypoxic environment, and for their resistance to radiation and chemotherapy also. VHL can be considered a gate-keeper gene and remains an independent prognostic factor for patients with stage I–III tumors after adjustment for gender, age, stage, grading, and symptomatic presentation; furthermore it represents a molecular target for new therapies (*Kopper et al., 2006; Yao et al., 2002*). Although the

histological grade and a variety of new molecular parameters have been shown of clinical utility, clear cell carcinoma prognosis is still based on evaluation of the clinical-pathological stage.
Patients with clear cell renal carcinoma have a poorer prognosis as compared with patients with papillary and chromophobe renal cell carcinomas. However, there is no significant difference in cancer-specific survival between patients with papillary and chromophobe renal cell carcinoma.

Papillary renal cell carcinoma

Papillary RCC account for approximately 10% of RCC in large surgical series. This tumor typically consists of a central fibrovascular core with epithelial covered papillae, although a compact tubular architecture or sheets of short papillae resembling glomeruli can be found. It is possible to distinguish two varieties: type 1 tumors, with papillae covered by small, scanty cytoplasm cells arranged in a single layer on the papillary basement membrane, often contain aggregates of foamy macrophages and scattered psammoma bodies (Fig. 6); type 2 tumors, with pseudostratification of voluminous epithelial cells, show an eosinophilic cytoplasm and a higher nuclear grade (Fig. 7). These tumors are usually multifocal and are frequently associated with sclerosis of adjacent non-neoplastic renal tissue. Immunohistochemically, papillary RCCs coexpress vimentin and epithelial markers and are often positive for CD-10 (93%) RCC antibody (93%) and S-100 protein (55%). Cytokeratin 7 and MUC1 immunohistochemical expression is more frequently seen in type 1 than type 2 papillary RCC (*Delahunt et al., 2005*).

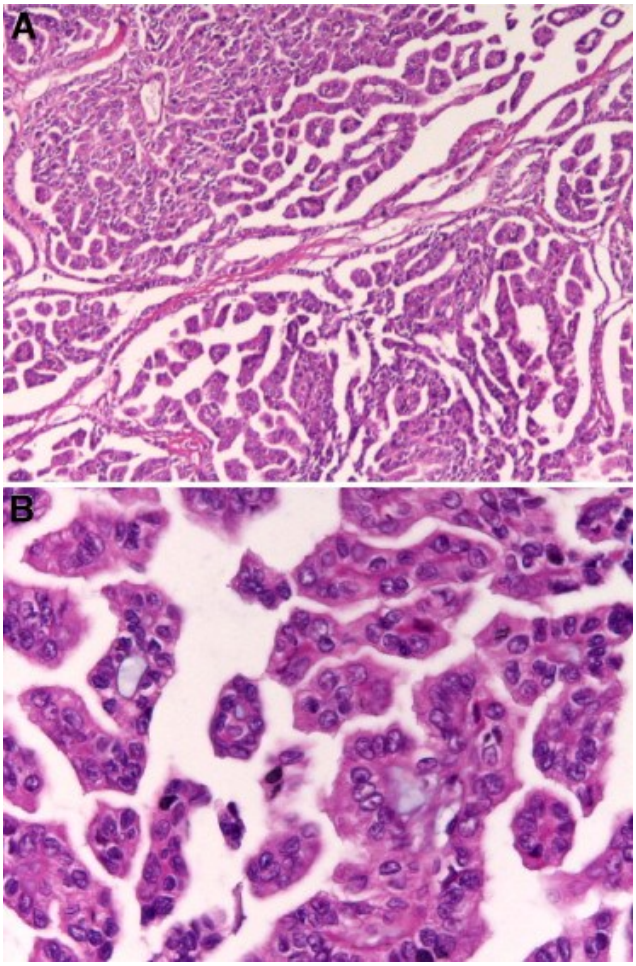


Figure 6: Malignant tumors. Papillary renal cell renal carcinoma type 1, 100X(A) and 400X(B), hematoxylin and eosin.

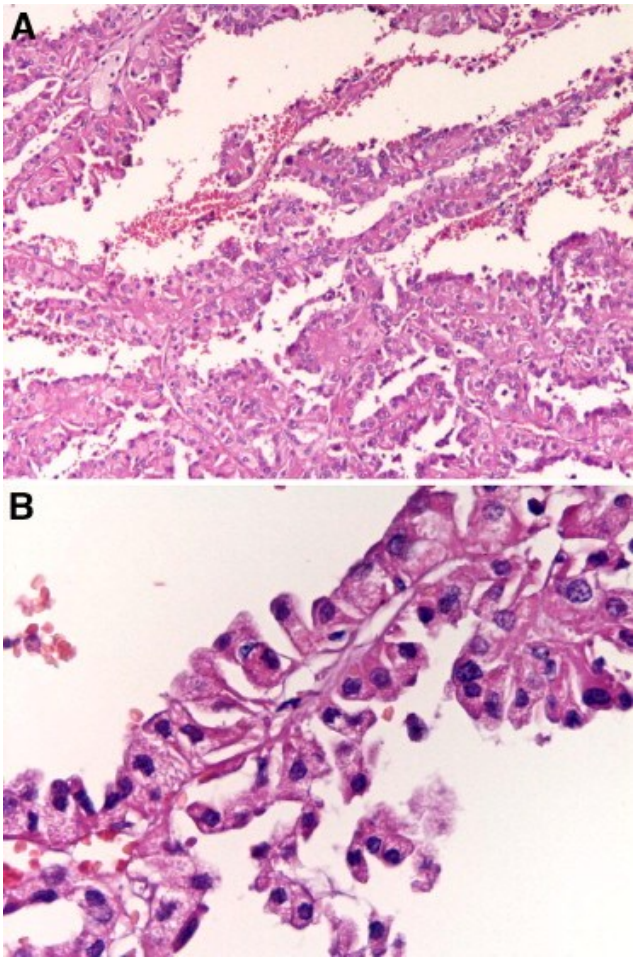


Figure 7: Malignant tumors. Papillary renal cell renal carcinoma type 2, 100X(A) and 400X(B), hematoxylin and eosin.

Zbar et al. showed the hereditary nature of papillary carcinoma: these forms had mutations of the MET protooncogene, and now we divide tumors into type 1 (small cells forming a single layer or the pure variant) and type 2 (large cells with pseudostratification and a solid architecture). Type 2 tumors tend to show a more unfavorable clinical outcome. Immunohistochemical and lectin histochemical studies have revealed both proximal and distal nephron phenotypes. Types 1 and 2 tumors differ in terms of genotype and clinical outcome. Type 1 tumors show gains of chromosomes 7p and 17p, and differing patterns of allelic imbalance at 17q and 9p there are between the two tumor types. Type 1 tumors are usually of lower nuclear grade and clinical stage than type 2 tumors, while longer post-treatment survival for patients with type 1 tumors result from multivariate analysis (*Delahunt et al., 2001*).

Chromophobe renal cell carcinoma

Before 1986, chromophobe RCCs were included in the class of clear cell RCC, assuming them to have a low histological grade and so a favorable outcome. The publication of detailed descriptions of similar tumors in nitrosamine-induced animal models led to the realization that this was a new

group of RCCs, later shown to have a low malignancy potential. As in carcinogen-induced chromophobe RCC in rodents, human chromophobe RCCs exhibit a wide histological spectrum, with typical balloon cells with an abundant granular pale cytoplasm, or sometimes tumors composed of smaller cells with a deeply eosinophilic cytoplasm, resembling those commonly associated with oncocytoma (*Tickoo et al., 2000*).

It may be difficult to differentiate between the eosinophilic variant of chromophobe RCC and oncocytoma, particularly in larger tumors, and a hybrid form of the tumor has been suggested to exist. This is supported by the fact that chromophobe RCC and oncocytoma may coexist in the kidney, in the form of multiple tumors in the same kidney (oncocytomatosis). Histologically, chromophobe RCC shows mostly a solid pattern of growth with abundant granular eosinophilia in the cytoplasm (Fig 8). Pathologists have learned to ignore the aggressive appearance of the nuclei of chromophobe cells because these tumors have a much better prognosis. About 10% of chromophobe RCC show eosinophilic cells and in such case features in favor of carcinoma over oncocytoma are:

- cellular discohesion in paraffin-embedded sections,
- wrinkling of the nuclear margin with an inconspicuous nucleolus,
- perinuclear cytoplasmic clearing (perinuclear halo),
- hyalinization of the walls of larger vessels,
- diffuse Hale's colloidal iron staining,
- presence of classical "balloon" chromophobe cells elsewhere in the tumor (sample widely),
- presence of amorphous calcific deposits but not psammoma bodies within the tumor interstitium (*Delahunt et al., 2005*).

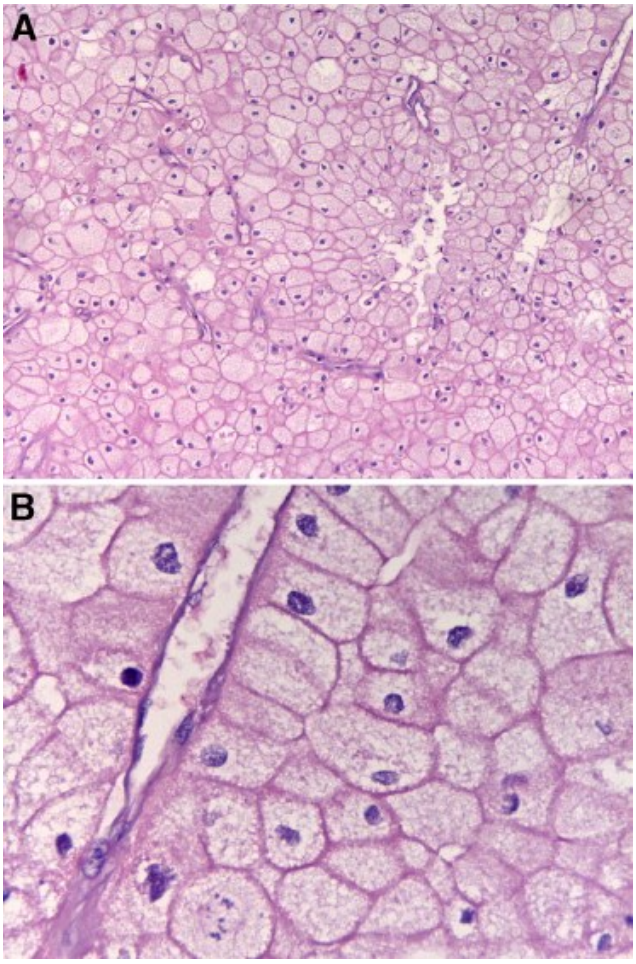


Figure 8: Malignant tumors. Chromophobe renal cell renal carcinoma, 100X (A) and 400X (B), hematoxylin and eosin.

So despite the fact that many of these tumors appear to be extracapsular (25%), larger than other RCC (about 9 cm) and that these tumors represent an extremely large group, they seem to have much better prognosis than clear cells, and also much better prognosis than papillary carcinomas. Based on the following facts, oncocytoma and chromophobe RCC are considered to be intimately related. First, both are considered to be derived from the intercalated cell of the collecting duct. Second, both are expected to have alterations of mitochondria, i.e., rearrangements of mitochondrial DNA and increased mitochondria in oncocytoma and numerous mitochondria-derived microvesicles in chromophobe RCC. Third, both are frequently observed in oncocytosis, with or without Birt-Hogg-Dubè (BHD) syndrome.

In addition, there are several reports of a hybrid tumor-composed mixture of oncocytic and chromophobe elements. Therefore, oncocytoma could be the benign counterpart of chromophobe RCC (*Mancini et al., 2008*).

Collecting duct carcinoma

The last, and most dangerous, type is collecting duct carcinoma (Bellini's carcinoma), a rare histotype of renal cancer. Its low incidence (<1% of RCC) makes the natural history difficult to define; however, the clinical behavior is often aggressive with an inauspicious prognosis. Advanced and metastatic disease is present in about 35% to 40% of patients and two thirds of them die within 2 years of diagnosis, because it is refractory to chemotherapy and immunotherapy. This rare tumor is characterized by pleomorphic cells arranged in irregular tubules within a desmoplastic stroma (Fig 9).

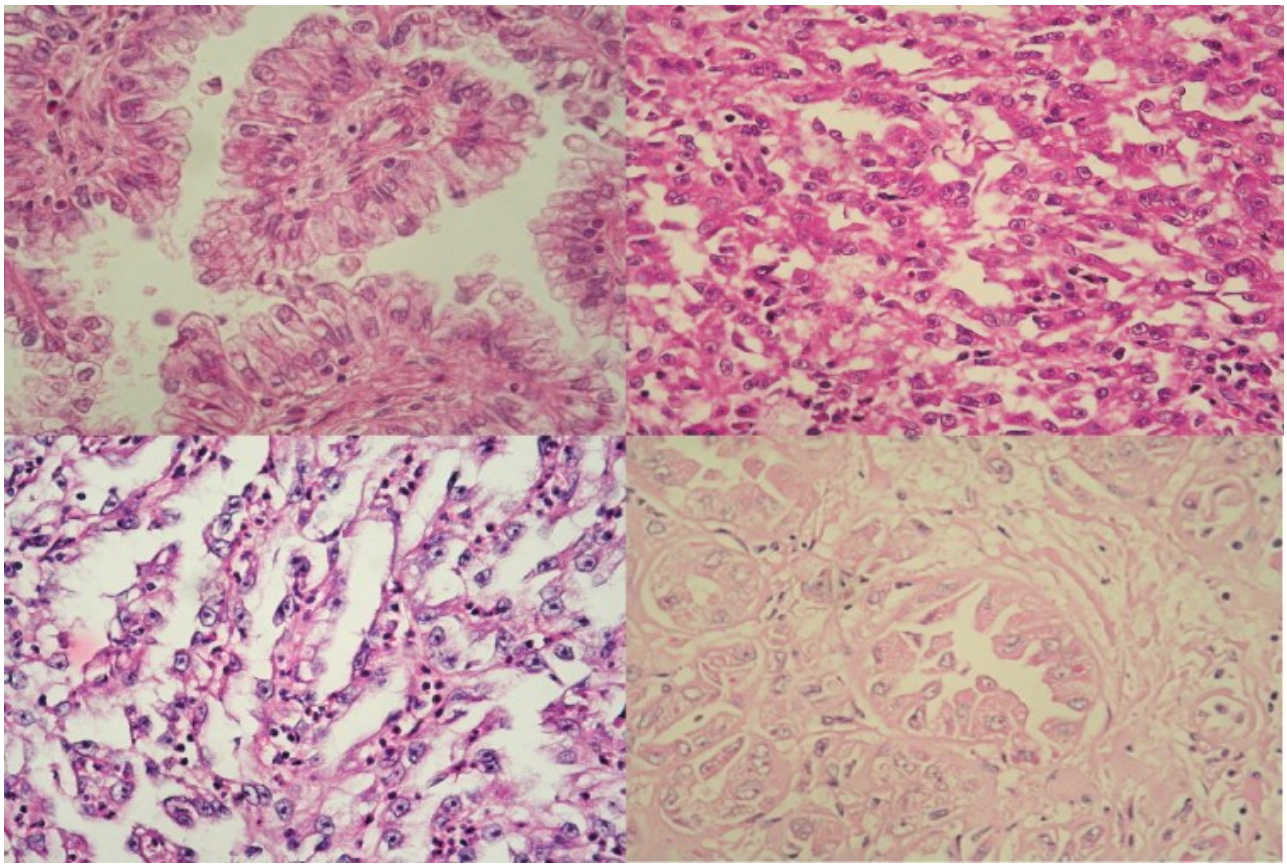


Figura 9: Malignant tumors. Collecting duct carcinoma: representative examples of 4 different cases of Bellini's carcinoma, hematoxylin and eosin (400X).

A cytological atypia has often been described in residual Bellini ducts near the tumor.

These tumors originate in the renal medulla but the site of origin is often unclear due to the advanced stage of the tumor at diagnosis. No peculiar set of chromosome alterations for collecting duct carcinoma has yet been identified due to its low incidence; this fact has likely led to an over-diagnosis of unclassifiable renal cancers, particularly in cases with complex morphological aspects.

1.5.2 Benign renal cell tumors

Papillary adenoma

This represents the most common epithelial tumor of renal tubules and is found in 10% to 40% of specimens. Histological alterations are similar to those of papillary RCC; it is usually solitary, small, with regular borders and a tubulopapillary architecture in the renal cortical parenchyma (*Henke et al., 2002)*.

Oncocytoma

Oncocytomas are benign tumors deriving from the epithelial intercalated cells of the kidney. They are usually diagnosed postoperatively due to differential diagnostic problems with renal cell carcinoma. Renal oncocytoma has several features that overlap those of other renal neoplasms with a preponderance of granular cytoplasm, such as chromophobe, granular, and papillary renal cell carcinomas. Histologically, renal oncocytoma is composed of an exclusive or predominant component of acidophilic cells with three architectural patterns: (1) the "classic" pattern, composed of a characteristic nested or organoid arrangement of cells, each surrounded by a distinct reticulin framework; (2) a "tubulocystic pattern" with numerous closely packed, cystically dilated tubular structures; and (3) a "mixed pattern," which has both the organoid and the tubulocystic patterns.

Cytologically, the neoplasms also showed a mixture of cell types, the most common being the classic oncocyte, which consisted of round or polygonal cells with a moderate to abundant granular, eosinophilic cytoplasm, and small round nuclei with evenly dispersed granular chromatin (*Mancini et al., 2008*).

Epithelioid angiomyolipoma

Epithelioid angiomyolipoma is a distinct subtype of angiomyolipomas strongly associated to tuberous sclerosis composed of clear or eosinophilic cells that can mimic other entities, particularly renal cell carcinomas and renal oncocytomas (*Mete et al., 2011*).

On gross examination, epithelioid angiomyolipomas may show hemorrhagic appearance and adipous tissue may not be apparent.

The tumor is totally or predominantly composed of epithelioid cells (Fig. 10). Epithelioid cells are polygonal in shape and show clear or eosinophilic cytoplasm with round to oval nuclei. There is no specific growth pattern. Frequently, conventional non epithelioid areas also appear in the tumor sampling.

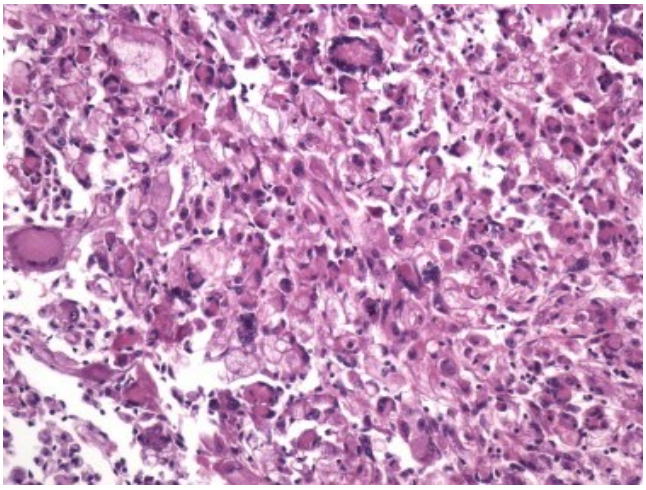


Figura 10: Epithelioid and giant cells showing no definite pattern of growth in an epithelioid angiomyolipoma.

1.6 THE ROLE OF MICRORNAs IN KIDNEY DISEASE

1.6.1 MicroRNAs in renal physiology

The suggestion of organ-specific roles for miRNAs emerged with the demonstration of tissue-restricted miRNA expression, including clusters of miRNAs that are expressed specifically in the kidney (*Sun et al., 2004*). Conversely, the absence or lower levels of particular miRNAs in the kidney compared with other organs may permit renal specific expression of target proteins that are important for kidney function (*Liu et al., 2004*). Examples of miRNAs that are more abundant in the kidney compared with other organs include miR-192, miR-194, miR-204, miR-215 and miR-216 (Table 4) .

High levels of expression in kidney
mir-192
mir-194
mir-204
mir-215
mir-216
Low levels of expression in kidney
mir-133a
mir-133b
mir-1d
mir-296
mir-1a

mir-122a
mir-124a

Table 4: Differential expression of miRNA in human kidney tissue.

Tian et al. (2008) established the first differential profile of miRNA expression between the renal cortex and medulla of rats indicating a potential role in tissue specification. However, cell type-specific miRNAs in the kidney have not yet been reported. A critical role of miRNA regulation in the progression of glomerular and tubular damage, and the development of proteinuria have been suggested by studies in mice with podocyte-specific deletion of Dicer (*Shi et al., 2008; Harvey et al., 2008; Ho et al., 2008*). All three reports showed major renal abnormalities in these mice including proteinuria, podocyte foot process effacement, glomerular basement membrane abnormalities, podocyte apoptosis, podocyte depletion and mesangial expansion. There was a rapid progression of renal disease with initial development of albuminuria followed by pathological features of glomerulosclerosis and tubulointerstitial fibrosis. This led to renal failure and death by 6–8 weeks. It is likely that these phenotypes are due to the global loss of miRNAs because of Dicer deletion, but given multiple miRNAs and their myriad targets, the precise pathways responsible require identification. These investigators also identified specific miRNA changes, for example, the downregulation of the miR-30 family when Dicer was deleted. Of relevance, the miR-30 family was found to target connective tissue growth factor, a profibrotic molecule that is also downstream of transforming growth factor. (*Duisters et al., 2009*). Thus, the targets of these miRNAs may regulate critical glomerular and podocyte functions. Recently, another study has shown that deletion of Dicer in the renin secreting cells of mice severely reduced the number of juxtaglomerular cells, decreased expression of the renin genes, lowered plasma renin concentration and decreased blood pressure (*Sequeire-Lopez et al., 2010*). The kidneys developed striking vascular abnormalities and prominent striped fibrosis. These findings highlight the important roles of Dicer and miRNAs in renal physiology and pathology, although the extent to which such genetic studies reveal an essential and fundamental role of Dicer in cellular function, as opposed to a specific role in renin secreting cells, is arguable. The importance of Dicer in cellular function is further highlighted by Wei's study. They established a mouse model with targeted Dicer deletion in renal proximal tubules. These mice had normal renal function and histology despite a global downregulation of miRNAs in the renal cortex. However, these mice were strikingly resistant to renal ischaemia-reperfusion injury, showing significantly better renal function, less tissue damage, lower tubular apoptosis and improved survival compared with their wild-type counterparts (*Wei et al., 2010*).

1.6.2 MicroRNAs implicated in kidney disease and cancer

Phatogenic miRNA mutations are extremely rare, having been identified only in cancer. Most polymorphisms involving miRNAs lie outside the mature sequence and potentially influence processing but not targeting. miRNA misexpression has been implicated in the pathogenesis of various kidney diseases and cancer (*Saal and Harvey, 2009*) (Table 5).

Pathology	***miRNA***	***Renal expression***	***Direct targets***	***Functional role***
Polycystic kidney disease	miR-15a	Not yet determined	Cdc25A	Proliferation
	miR-17	Not yet determined	PKD2	Proliferation
Diabetic nepropathy	miR-21	Glomeruli and interstitium	PTEN	Inhibition of apoptosis
	miR-23b	Podocytes and tubules	Not yet determined	Modulation of TGF-β1 signaling
	miR-192	mesangium	SIP1	Collagen synthesis
	miR-216	mesangium	YB-1	Modulation of TGF-β1 signaling
	miR-377	mesangium	PAK1/SOD1/SOD2	Fibronectin synthesis
Kidney cancer	miR-34a	Tumor cells	Sirt1	apoptosis
	miR-141/200	Tumor cells	ZEB2	Epithelial to mesenchymal transition
	miR-438-3p	Tumor cells	BBC3	apoptosis

Table 5: MicroRNAs linked to kidney disease and cancer.

Diabetic nephropathy

Diabetic nephropathy is the leading cause of end-stage kidney disease but the understanding of the disease mechanisms is incomplete. Studies of miRNA expression in diabetic nephropathy have so far emerged predominantly from animal models of diabetes and the effects of hyperglycaemia (*Li et al., 2010*).

In one study, miR-192 levels were shown to be increased in glomeruli isolated from streptozotocin-injected diabetic mice and diabetic mice db/db when compared with non-diabetic mice (*Kato et al., 2007*). In this study, miR-192 was shown to regulate E-box repressors that are responsible for controlling the expression of TGF-β-induced extracellular matrix proteins, collagen 1-α 1 and 2 (Col 1α1 and 2). Col 1α1 and 2 were shown to accumulate during diabetic nephropathy; therefore, these results suggest a potential role of miR-192 in diabetic nephropathy or that miR-192 can be an effector of TGF-β. However, discordantly a recent study demonstrated that miR-192 expression decreased in proximal tubular epithelial cells in response to TGF-β (*Krupa et al., 2010*). The loss of miR-192 correlates with tubulointerstitial fibrosis and reduction in eGFR in renal biopsies from patients with established diabetic nephropathy. This suggests that mesangial cell and proximal tubular epithelial cell miRNA expression may exhibit different responses to TGF-β.

Recently, Akt kinase, a key mediator of diabetic nephropathy, was found to be activated through downregulation of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), which is targeted by miR-216a and miR-217. In turn, these miRNAs are upregulated by TGF-β, and indirectly by miR-192, in mouse mesangial cells (*Xin et al, 2007*). In other animal studies, *Zhang et al.* showed miR-21 expression was downregulated in response to early diabetic nephropathy in vitro and in vivo. Overexpression of miR-21 inhibited proliferation of mesangial cells in high-glucose condition. The 24h urine albumin excretion rate of diabetic db/db mice decreased after exposure to elevated miR-21. The same study also identified PTEN as a target of miR-21 (*Zhang et al., 2009*).

Another study has reported overexpression of miR-377 in human and mouse mesangial cells when exposed to high glucose levels (*Wang et al., 2008*). MiR-377 has been demonstrated to reduce the expression of p21-activated kinase (PAK1) and manganese superoxide dismutase (mnSOD). This enhances fibronectin production, which is characteristic of mesangial cells in diabetic nephropathy.

Likely, many other miRNAs expressed in podocytes, tubular and other renal cells will be deregulated under hyperglycaemic conditions.

In diabetic nephropathy, alteration of miRNA expression in response to several pathophysiological states is of interest, notably hypoxic-ischaemic and hyperglycaemic stimuli. The findings by Wang and colleagues have already provided the first glimpse of the effects of hyperglycaemia on miRNA

expression in mesangial cells. In addition, hyperglycaemia has been found to affect endothelial dysfunction through miR-221 (*Li et al., 2009*).

Polycystic kidney disease

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited renal diseases. Genetically, mutations in the polycystic kidney disease-1 gene (PKD1) account for 85% of ADPKD; whereas mutations in the polycystic kidney disease-2 gene (PKD2) are responsible for the remainder. PKD2 encodes a protein termed polycystin-2. Aberrant expression of polycystin-2 causes abnormal proliferation of renal tubular and biliary epithelial cells, eventually leading to cystogenesis (*Wu et al., 2002*). The potential role of microRNAs in control of expression of PKD genes and in mediating functional effects has recently been explored. Two groups have demonstrated that miR-17 directly targets the 3′UTR of PKD2 and post-transcriptionally represses the expression of PKD2 (*Tran et al., 2010*). Moreover, they also showed that overexpression of miR-17 may promote cell proliferation via post-transcriptional repression of PKD2 in HEK293 T-cells. Using a rat model of PKD, 30 differentially expressed miRNAs have been identified in diseased kidney tissues compared with healthy rat, 29 of which are downregulated (*Pandey et al., 2008*). The deregulated miRNAs in PKD were associated with genes in 24 functional categories, including several pathways important to cyst formation such as mTOR signalling, mitogen-activated protein kinase signalling, Wnt signalling and TGF-β pathway (*Pandey et al., 2008*). However, these correlations require experimental validation. MiR-15a has been reported to modulate the expression of cell cycle regulator Cdc25A and affect hepatic cystogenesis in a rat model of PKD (*Lee et al., 2008*). In situ hybridization suggested that miR-15a was downregulated in liver tissues of patients with ADPKD, autosomal recessive PKD or congenital hepatic fibrosis, as well as rats with PKD. Conversely, overexpression of miR-15a in cells derived from the PKD rat led to a decrease in Cdc25A protein, small decreases in G1-S phase transition and cellular proliferation, and a larger drop in cyst growth in vitro. This disproportionate effect on cyst growth suggests that decreased miR-15a may promote cystogenesis through alternate mechanisms in addition to increased cell proliferation.

Other kidney diseases

In trying to understand the role of microRNAs in renal diseases an obvious approach has been to compare microRNA expression between samples from normal and affected patients. In renal disease, such studies have included patients with IgA nephropathy, lupus nephritis, hypertension and renal cancer. A study by *Dai* and colleagues compared miRNA expression of IgA nephropathy

biopsy samples from 11 patients with three control patients (*Dai et al., 2008*). They were able to identify 132 miRNA in both patients with IgA nephropathy and normal control renal tissue samples, of which 31 miRNAs were downregulated and 35 upregulated in diseased tissues.

More recently, another study has reported differential intrarenal expression of miR-200c, miR-141, miR-205 and miR-192 in IgA nephropathy and findings correlated with disease severity and progression (*Wang et al., 2010*). The deregulated expression of miR-200c and miR-205 is of particular interest given their link with epithelial-to-mesenchymal transition (EMT). Sixty-six miRNAs have also been found to be differentially expressed in a small number of human kidney tissues from patients with Class II lupus nephritis as compared with healthy control subjects (*Dai et al., 2006*). Differential expression of miRNAs (16 miRNA, 7 downregulated and 9 upregulated) in peripheral blood mononuclear cells (PBMC) has also been reported in patients with systemic lupus erythematosus when compared with normal healthy subjects. In a recent study, several other miRNA, miR-200a, miR-200b, miR-141, miR-429, miR-205 and miR-192, were increased in kidney biopsy samples from patients with hypertensive glomerulosclerosis (*Wang et al., 2010*).

Differential miRNA expression has also been linked to both renal and transitional cell carcinomas (*Huang et al., 2009*). Hypoxia-regulated miRNAs, such as miR-210, have been found to be expressed differentially in renal cell carcinomas and may have implications for tumour pathogenesis (*Chow et al., 2010*). Similarly, an oncogenic cluster of miRNAs has been implicated in Wilms tumour (*Kort et al., 2008*).

1.6.3 MicroRNAs and their target gene networks in renal cell carcinoma

RCC is known to be characterized by the loss of the VHL gene. This tumor suppressor gene localizes to chromosome 3p25.3. It acts to prevent tumor growth and is involved in regulating cellular signaling induced by hypoxia. Chromosomal locations of miRNAs target genes have frequently provided important insight into the roles of miRNAs specific diseases. Under normal oxygen pressure, the VHL gene binds to the a subunit of hypoxia inducible factors (HIFs), inducing their poly-ubiquitinylation and subsequent degradation in the proteasome (*White et al., 2010*). Mutations of the VHL gene or even its loss will lead to a downstream signaling cascade of events that trigger hypoxia-like cellular processes under normal oxygen status and cause an increase in cellular proliferation leading to cancer progression. HIF-1α is one of the key regulators of hypoxia response and transcription factors, allowing the regulation of many genes and maintaining steady cell survival under low oxygen levels (*Chow et al., 2010*). It has been well documented that several miRNAs are downstream effector molecules of the HIF-induced hypoxia response.

For example, the expression of miR-210 increases under hypoxic conditions and can target the iron-sulfur cluster protein (ISCU) which is involved in the mitochondrial electron transport chain, suggesting a potential mechanism for regulating anaerobic respiration in tumors (*White et al., 2010*). MiR-210, which serves as a powerfull prognostic marker in mammary carcinomas, could also link hypoxia and cell cycle in cancer by its regulation of e2f transcription factor 3 (e2f3), a key protein in cell cycle (*Giannakakis et al., 2008*). MiR-210 upregulation has been well documented also in lung, prostate and liver cancer (*Chow et al., 2010*). Other hypoxia related miRNAs being at the top according to the most significant fold changes in tumor tissue are up-regulated miR-155 and miR-21, whereas in case of miR-21, SLC12A1 and TCF21 were identified as target molecules in RCC (*Liu et al., 2010)*. miR-155 and miR-21 together with miR-210 can furthermore reduce proapoptotic signaling in response to a hypoxic environment established by VHL loss, and are consistently overexpressed in a variety of human tumors (*Ambs et al., 2008*). There are also implications that up-regulated miR-210, -106a, -21 and -27a are induced in low oxygen levels due to tumor growth, and through translational repression of their target genes, have downstream biological impacts on cell survival and/or proliferation (*Cho et al., 2007*).

MiRNAs contribute to the RCC development at different levels and it is evident that miRNAs can target various altered signaling pathways related to RCC pathogenesis (*Redova et al., 2011*) (Fig. 11).

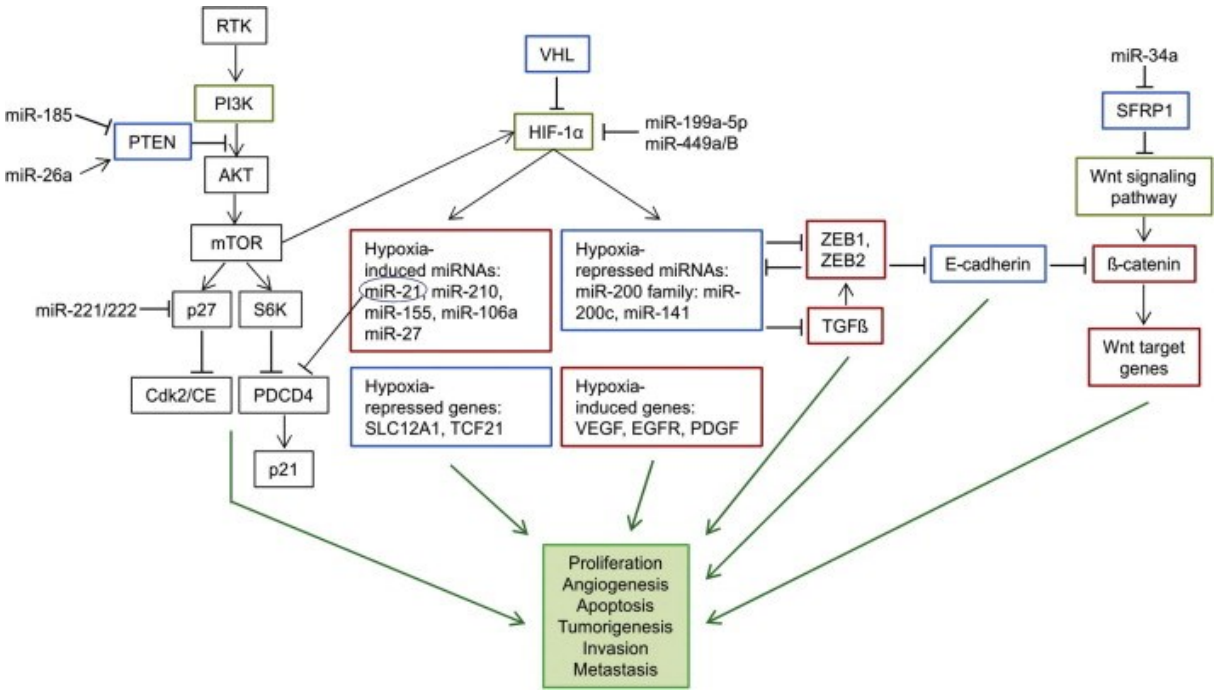


Figura 11: Model of dysregulated patways in RCC based on predicted miRNA/mRNA interactions and known signalling pathways. Blue and red indicate down-regulated and up-regulated genes in tumors, respectively, olive green shows pathways.

1.6.4 MicroRNAs in human kidney cancer subtypes

Renal neoplasms are a group of heterogeneous tumors with distinct histological, biochemical and molecular genetic characteristics, which not only display different prognosis but also require different clinical management (*Takahashi et al., 2003*). Therefore, it is important to study the miRNA expression patterns of different subtypes of renal cell neoplasms.

Petillo et al. (2009) observed a number of miRNA expression shifts which may be associated with development of specific tumor subtypes, progression from benign to malignant phenotype, or tumor progression. For examples, the overexpression of miR-424 and miR-203 in ccRCC relative to papillary, as well as the inversion of expression of miR-203 in the benign oncocytoma (where it is underexpressed relative to normal kidney) as compared to the malignant chromophobe RCC (where it is overexpressed relative to normal kidney). Also investigated miR-203 expression, finding that its expression is increased in malignant chromophobe RCC samples, but decreased in the begnin oncocytoma samples. An unproven hypothesis exists that oncocytomas can progress into malignant chromophobe RCC. If this proves to be the case, it may be that miR-203 is one of the factors either driving or resulting from this progression by an epigenetic mechanism or by other biological processes.

2. MATERIALS AND METHODS

2.1 Cell Cultures

Have been used the following human renal cells:

- KJ29: Non papillary human renal carcinoma cell line;
- 4/5: human tubular epithelial cell.

These cells were grown in 50% D-MEM (*Dulbecco's Modified Eagle's Medium*) and 50% F12 (*Ham's nutrient mixture*), supplemented with 10% FBS (fetal bovine serum), streptomycin (50 mg/mL) and penicillin **(**100mg/mL). Cells are manteined in culture in a humidified incubator and thermostated at 37°C in presence of 5% CO_2.

Cytogenetic analysis has shown that KJ29 have a modal chromosome number of 50 with some marker chromosomes, including rearrangements of cf chromosomes 1 and 3. The antigenic phenotype is characterized by co-expression of cytokeratin and vimentin, as well as expression of urothelium differentiation antigens, low levels of class II MHC antigens and no class I antigens. The cell line wich is highly tumorigenic in athymic mice displays expression of erb B-2 and c-met oncogenes and high expression of cell-cycle related and Ha-ras 1 genes (*Barletta C., et al 1995*).

2.2 RNA extraction

2.2.1 Cells and tissues after nephrectomy

Total RNA from pelleted cells or homogenized tissues was extracted by TRIZOL treatment (Invitrogen SRL, Milano, Italy). After the addition of chloroform (0.2 mL), shaking samples for 15s, they were centrifuged for 15 min at 12000 g at 4°C. After centriguation, the aqueous phase was collected and RNA precipitated with isopropanol at 8500g for 20 minutes, 4°C. After two washes with 75% ethanol, RNA was checked by electrophoretic analysis on a 0,8% agarose gel and quantified by spectrophotometric analysis.

2.2.2 Paraffin-embedded tissues

RNA extraction from tissues was performed with *"RecoverAll Total Nucleic Acid Isolation Kit"* (Ambion, Milano, Italy). 1 mL of xilene 100% to tissue slices (four replicates from 20 μm), and heated for 3 min a 50°C to melt the paraffin. After centrifugation at 12000 g for 2 min at room temperature, the xilene was removed without disturbing the pellet. After washing the pellet twice with 1 mL 100% ethanol, the pellet was dried in a centrifugal vacuum for at 40°C for 20 min. Then, 200 μL *"Digestion Buffer"* and 4 μL *"Protease"* were added to each sample, and the sample incubated in the heat block for 30-60 min at 50°C, then 15 min at 80°C. In order to isolate RNA, *"additive-ethanol"* mixture was prepared, loaded in the *"Filter Cartridge"*, and it was centrifuged

at 10000g for 30 sec. Two washes with 700 µL of *"Wash2/3"* were carried out, and was added 60 µL *"DNase mix"* and incubated for 30 min at room temperature. Finally, after washing the filter with 700 µL *"Wash1" and 500* µL *"Wash2/3"*, RNA was eluted with 60 µL *"Eluition Solution"* and centrifuged for 1 min at maximum speed to pass the mixture through the filter. RNA was stored at -80°C.

2.3 SYNTHESIS OF cDNA

Synthesis of cDNA was carried out using the kit "*TaqMan MicroRNA Reverse Transcription Kit*" (Applied Biosystems, Monza, Italy), and specific primers: RT-RNU6B e RT-has-miR501-5p. The RT reaction took place in a volume of 15 µL: 7 µL of RT *Master Mix* (0,15 µL of *dNTP mix* (100 mM), 1 µL *RT enzyme* (50U/ µL), 1,5 µL *RT buffer* (10X), 0,188 µL *RNase Inhibitor* (20U/ µL), 4,162 µL *Nuclease free water*), 250 ng RNA and 3 µL primer. Samples were loaded in a thermal cycler at 16°C for 30 min and at 42°C for 30 min.

2.4 REAL TIME PCR

Real Time quantitative PCR (qPCR) was very similar to traditional PCR. The major difference being that with qPCR the amount of PCR products is measured after each round of amplification while with traditional PCR, the amount of PCR product is measured only at the end point of amplification.

Amplification products were measured as they were produced using a fluorescent label. During amplification, a fluorescent dye binds, either directly or indirectly via labelled hybridizing probe, to the accumulating DNA molecules, and fluorescence values are recorded during each cycle of amplification process. The fluorescence signal is directly proportional to DNA concentration over a broad range, and the linear correlation between PCR product and fluorescence intensity is used to calculate the amount of template present at the beginning of the reaction. The point at which fluorescence is first detected as statistically significant above the baseline or background, is called the *threshold cycle* or *Ct Value*.

The *Ct Value* is the most important parameter for quantitative PCR. This threshold must be established to quantify the amount of DNA in the samples. In theory, an equal number of molecules are present in all of the reactions at any given fluorescence level. Therefore, at the threshold level, it is assumed that all reactions contain an equal number of specific amplicons.

Real-time PCR system exploits fluorogenic-labeled probes that use the 5´ nuclease activity of Taq DNA polymerase. TaqMan probes are dual labelled, hydrolysis probes that increase the specificity of real-time PCR assays. TaqMan probes contain:

- a report dye (FAM dye) linked to the 5´ end of the probe,

- a nonfluorescent quencher (NFQ) at the 3′ end of the probe,
- MGB moiety attached to the NFQ.

TaqMan MGB probes also contain a minor groove binder (MGB) at the 3′ end of the probe. MGBs increase the melting temperature (T_m) without increasing probe length.

The probe is constructed with a fluorescent reporter dye bound to the 5′ end and a quencher greatly reduces the fluorescence emitted by reporter dye by fluorescence resonance energy transfer through space (Figure 12). If the target sequence is present, the probe anneals between primer sites and is cleaved by the 5′ nuclease activity of the Taq DNA polymerase during extension. This cleavage of the probe separates the reporter dye from the quencher, increasing the reporter dye signal; and removes the probe from the target strand, allowing primer extension to continue to the end of template strand.

Amplification reactions were carried out using the system *ABI Prism 7700 Sequencer Detector* (Applied Byosistem, Monza, Italia). This instrument consists of a *Thermal Cycler, ABI Prism 7700,* connected to a computer, where the *software Sequence Detection Application Program 1.6.3.* was installed.

To quantify the target sequence the comparative C_T ($\Delta\Delta$CT) method (relative quantification) was used (Livak et al., 2001). The *small nuclear* U6B (reference gene) was used as endogenous control for the normalization of the samples. The ΔCT value was calculated as follows:

$\Delta C_T = C_T$(target gene) – C_T(reference gene). The highest ΔC_T that did express the lowest amount of RNA was identified as the calibrator. Its value was subtracted from the ΔC_T values of the samples to determine the $\Delta\Delta C_T$ value: $\Delta\Delta C_T = \Delta C_T$(sample) – ΔC_T(calibrator). Final results, were determined as follows: Fold change $= 2^{-\Delta\Delta CT}$.

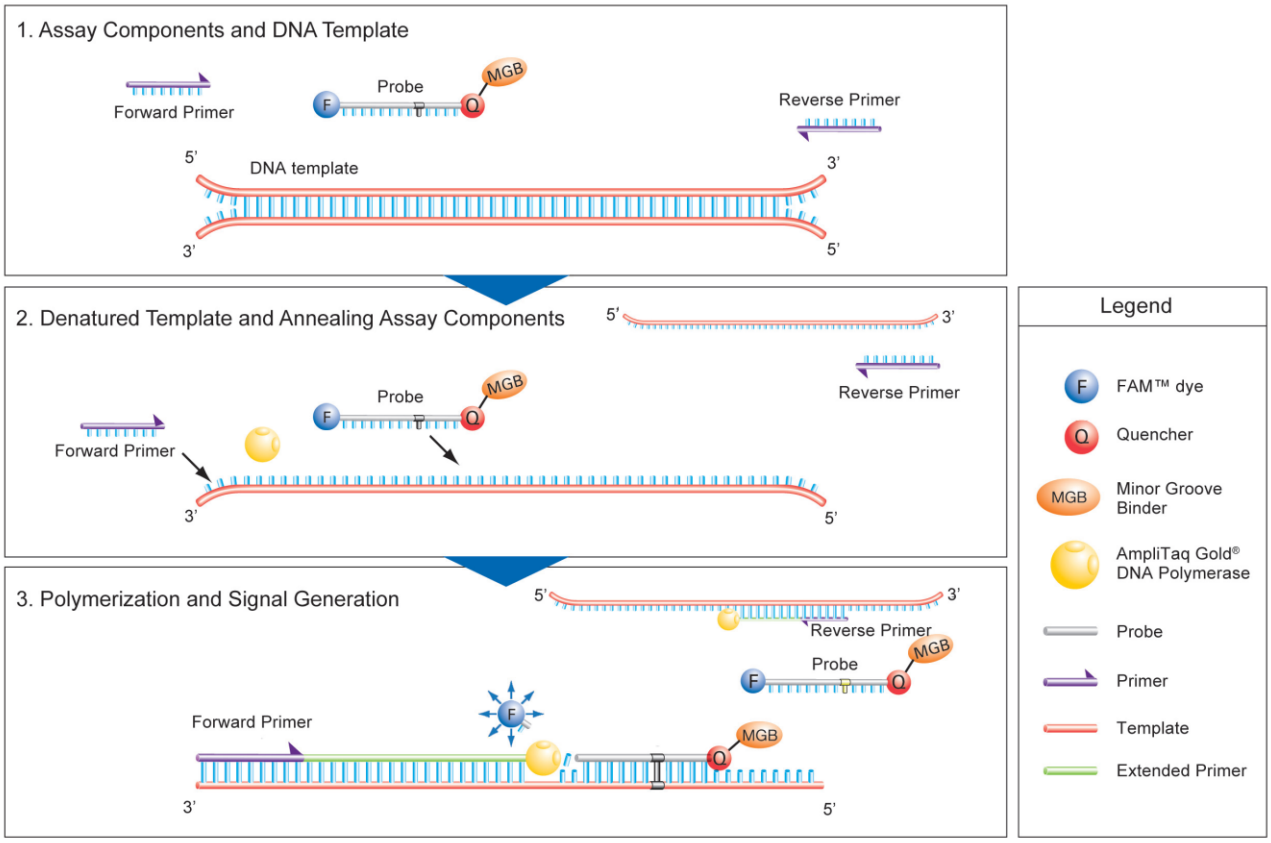


Figura 12: TaqMan Gene Expression Assay reaction steps.

2.5 WESTERN BLOTTING

Detection of 42/44kDa p-ERK/ERK, p53, MDM2, actin was performed on total cell lysates.

2.5.1 Cellular total extract

To obtain total lysate, cells in T25 confluent flasks or sub-confluent 6-well plates were washed twice with D-PBS containing protease inhibitors. After mechanical detachment, cells were harvested and centrifuged at 1500rpm for 5 minutes, 4°C. The pellet or the homogenized tissue, was resuspended in a single detergent lysis buffer (10mM TRIS-HCl pH 7.5, 10 mM NaCl, 3 mM MgCl2, 1% v/v triton X-100 and complete protease inhibitor cocktail) for 30 minutes on ice. After centrifugation at 12000g for 3 minutes 4°C, an amount of cell lysate was tested for protein content by Bradford method.

2.5.2 Electrophoresis and immunoblotting

The total lysate, premixed with 6X loading buffer (60mM Tris pH 6.8, 1.8% SDS, 6% v/v glycerol, 0.6M DTT, 0.002% p/v bromophenol blue), was subjected to 8% SDS–PAGE. Proteins were transferred to nitrocellulose membrane (Pierce) through elettroblotting for 2 hours at 100 mA and 200 Volts, in transfer buffer (25mM Tris, 192mM Glycine, 20% methanol pH 8.3). Blots were

blocked for 90 minutes at room temperature in 5% non-fat dry milk in 1X TBS, 0.05% Tween20®. Then, membranes were processed for immunoblotting with the monoclonal primary antibody, diluted 1:1000 in 5% non-fat dry milk in 1X TBS, 0.05% Tween20®, over night. After this incubation, three washes with 1X TBS 0.05% Tween20® were performed and consequently nitrocellulose membranes were incubated in 5% non-fat dry milk with the secondary anti-Ig**G** HRP-coniugated antibody (1:10000) for 60 min at room temperature. After three washes with 1X TBS 0.05% Tween20®, immuno-bands were visualized by autoradiography with the enhanced chemioluminescence system (SuperSignal West Femto o SuperSignal West Pico, Pierce). Band intensity was measured with the Model GS-700 Imaging Densitometer (BioRad).

2.6 TRANSFECTION WITH MIR 501-5p AND ANTAGOMIR

AntagomiR-501-5p (*Ambion*) and MiR-501-5p (*Origene*) transfection was made with kit *TurboFect Transfection Reagent* (Fermentas), a solution of a cationinc polymer in water. The polymer forms compact, stable, positively charged complexes with DNA. These complexes protect DNA from degradation and facilitate gene delivery into cells. Transfection occurred in KJ29 and 4/5 cells; a mixture was prepared containing DMEM, TurboFect, vector miR-501-5p or antagomiR, and allowed 20 min at room temperature. Finally, the mixture was added into each well.

2.7 CELL CYCLE ANALYSIS

KJ29 cells were plated at the density of $2x10^5$ for well, starved for 24h in 0,4%BSA and then transfected with antagomiR (*TurboFect Transfection Reagent*). After 24h, cells were collected, centrifuged at 1200 rpm for 5 min, washed with 1mL PBS and centrifuged for 5 min at 1200 rpm. Cell pellets were resuspended in 400µL *Propidium Iodide* (PI) solution for 30 min at 4C° in the dark. Therefore cell cycle was analyzed by *Becton-Dickinson FACS Calibur Immunocytometry System*. 20000 events were collected for each analysis; amount of cells in G0-G1, S or G2-M phase were expressed as percentage of PI positive counted cell (Software Cell Quest Pro, Becton Dickinson).

2.8 APOPTOSIS ANALYSIS

2.8.1 HOECHST STAINING

KJ29 cells (200000) were plated on 24 mm coverslips, starved 24h with 0,4%BSA. Apoptosis was evaluated after transfection with antagomiR or miR501-5p vector. Then, cells on coverslip were fixed with a 10% formalin solution, permeabilized with 0.1% Triton X100, 2% BSA in PBS, and stained with Hoechst 33258 (10mg/ml). Before analysis, cells were washed twice with D-PBS and

fluorescence at 510-540nm was recorded by a fluorescence microscope (Zeiss Axiovert200) equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, USA).

2.8.2 CASPASE-3 ASSAY

Caspase 3 activity was evaluated using EnzChek® caspase-3 Assay Kit (Invitrogen), which allows the detection of apoptosis by assaying for increase in caspase-3. 200000 cells for well were plated and starved 24h with 0.4% BSA. After transfection with antagomiR (*TurboFect Transfection Reagent*), cells were treated with 0.4%BSA for 24h. Then, cells were lysed for 30 min on ice, according to the manufacturer instruction. After centrifugation, 50µl of supernatant were incubated with 50µl of *2X substrate working solution* containing 5mM Z-DEVD-R110. Fluorescence was measured every min for 90 min at 520nm by a microplate reader (SpectraFluor Plus, TECAN). Values were normalized to the amount of protein samples by Bradford method.

2.9 PROLIFERATION CELLS ANALYSIS

Cell proliferation analysis was performed plating 5000 cells in 96-well plates and their starving for 24 h in DMEM/F12 supplemented with 0.4% BSA. Subsequently, cells were transfected with antagomiR and miR 501-5p vector (1 µg, 1.5 µg, 3 µg DNA) in absence of serum for 24 h, and maintained in culture for 24 h, 48 h and 72 h in DMEM/F12 1% FBS.

Cell proliferation was calculated by direct cell counting after trypan blue staining, using a Burker chamber and by a colorimetric method. The latter consists in the quantitation of formazan, a colored compound produced by the cells through the bio-reduction of tetrazolium salts added in culture medium. The amount of formazan, detectable recording the absorbance at 490 nm with a 96-well plate reader, is directly proportional to the number of living cells (CellTiter cell proliferation assay, Promega).

2.10 IMMUNOFLUORESCENCE

KJ29 cells (200000) were plated on 24 mm coverslips in 0.4%BSA, and after 24h were transfected with antagomiR. Subsequently, cells were fixed for 20 min in 0.4% formalin, washed three times in PBS buffer and permeabilized for 10 min in a PBS solution containing 0.2% Triton X-100. Then, cells were washed twice with PBS buffer and incubated at room temperature for 1h with anti-p53 monoclonal antibody (Pab-1801) (Santa Cruz Biotechnology, Italy) at a dilution of 1:500 in a solution containing 0.2% gelatine. After three washes with PBS, cells were treated with a 0,2% gelatine PBS solution, which contained secondary anti-mouse Alexa Fluor 594-conjugated antibody at room temperature for 1h in the dark, at a dilution of 1:1000. After other three washes in PBS,

cells were analyzed using a Zeiss Axiovert 200 fluorescence microscope equipped with a back-illuminated CCD camera (Roper Scientific, Tucson, AZ, USA).

2.11 IMMUNOPRECIPITATION

KJ29 cells were plated at the density of $2x10^5$ for well, starved for 24h in 0,4%BSA and then transfected with miR501-5p plasmid (*TurboFect Transfection Reagent*). After 24h, cells were collected, centrifuged at 1200 rpm for 5 min, washed with 1mL PBS inihibitors and centrifuged for 5 min at 1200 rpm at 4°C. Cell pellets was resuspended in 50µL of ice cold TBS, then were added 60 µL of TBS containing 2% SDS, and mixed quickly. Cell pellets was denaturated by heating for 10 min and placed immediately on ice for 5 min. Subsequently were added 900 µL of TBS containing 1% TritonX-100, centrifuged for 5 min at 10000 rpm at 4°C and discarded the pellet. At this point was added Protein G beads (50 µL) to the supernatant and incubated at 4°C for 1 h on rotator. After this time, was collected the supernatant after centrifugation (10000 rpm, 4°C, 1 min), was added anti-ubiquitin (Dako) and incubated at 4C° overnight on rotator. Then was added Protein G beads (50 µL) to the supernatant, and incubated at 4 C° for 1 h on rotator; washed the beads three times with 0.5 mL of TBS containing 1% Triton X-100 and centrifuged (10000 rpm, 4°C, 1min). Finally, was added 2x Laemli buffer to the beads, boiled at 98°C for 5 min, centrifuged (10000 rpm. 25°C, 2 min) and applied the supernatant to SDS-polyacrylamide gel.

All buffers need to be supplemented with proteosome inihibitors (10 µM MG132) and 10 mM N-ethylmaleimide.

2.12 STATISTICAL ANALYSIS

Analysis of data was performed using Student's t test (unpaired analysis). Differences were considered significant at a value of $p < 0.05$. All data were reported as mean ± SD (standard deviation) of at least three independent experiments in duplicate.

3. AIM

MicroRNAs, small noncoding RNAs, regulate gene expression at post-transcriptional level and, are involved in many biological processes including differentiation, proliferation and cell death. In addition to their physiological functions, miRs are found to be aberrantly expressed or mutated in many carcinomas including renal tumors, therefore they could play an oncogenic or tumor suppressive role in cancer cells. For this reason, in the last years, was planning to use microRNAs as both diagnostic and prognostic markers and as targets for new therapeutic strategies. Moreover, in kidney cancer would be crucial the identification of new more informative prognostic biomarkers because, currently, no specific therapy for renal carcinoma is available. In this regard, we have found that miR501-5p is randomly expressed in RCC tissues and it could have a possible role of molecular biomarker for kidney carcinoma. To verify the potential prognostic of miR501-5p, its expression will be associated with the outcome of patients with renal cancer in at least 5 years follow up studies. Moreover, mTOR signalling will also investigated because the mRNA of TSC1, a member of mTOR inhibitor complex, is a target of miR501-5p. Furthermore, it is known that mTOR signaling is abnormally activated in many kidney tumours with a poor prognosis.

Thus, the purpose of this thesis is to investigate the expression of miR501-5p and the activation of mTOR signal in kidney cancer tissues as well as in kidney cell lines depleted or enriched in miR501-5p sequences in order to identify new possible markers for renal carcinoma. This study aims to understand if high or low miR501-5p expression levels can inhibit or slow cellular processes involved in development and growth of kidney cancer.

4. RESULTS AND DISCUSSION

A study performed by microarray in autosomal polycystic kidney disease (ADPKD) subjects, the main research field of our laboratory, showed an increased expression of miR501-5p in ADPKD tissues compared with normal kidney (data not shown). Based on the iperproliferative nature of ADPKD, we have analysed the expression of this miR also in kidney carcinoma in particular for the easier availability to collect kidney cancer samples. In fact, since several years we collaborate with the Urology and Pathology Units of Sant'Anna hospital in Ferrara which provide us fresh frozen- and paraffin embedded-tissues of patients with renal carcinoma from the province of Ferrara, respectively. This collaboration allowed us to study the role of MicroRNA in renal carcinomas.

4.1 MicroRNA501-5p expression in renal carcinomas.

MiR501-5p expression was evaluated in 63 patients with clear cell renal cell carcinoma (ccRCC) and in 18 patients with papillary carcinoma (pRCC). MiR501-5p analysis was conducted by Real Time RT-PCR in both fresh and paraffin embedded tissues collected after surgical resection. Data were expressed as fold change calculated from the ratio among ccRCC or pRCC sample and normal tissue histologically identified. As shown in figure 13A, the expression of miR501-5p in ccRCC tissues is 2.7 fold increase compared with control. Conversely, in pRCC tissues the expression of miR-501-5p is 0.56 fold versus control (Figure 13B). These findings suggest that the expression of miR501-5p appears to be upregulated in clear cell cancer and downregulated in papillary carcinoma.

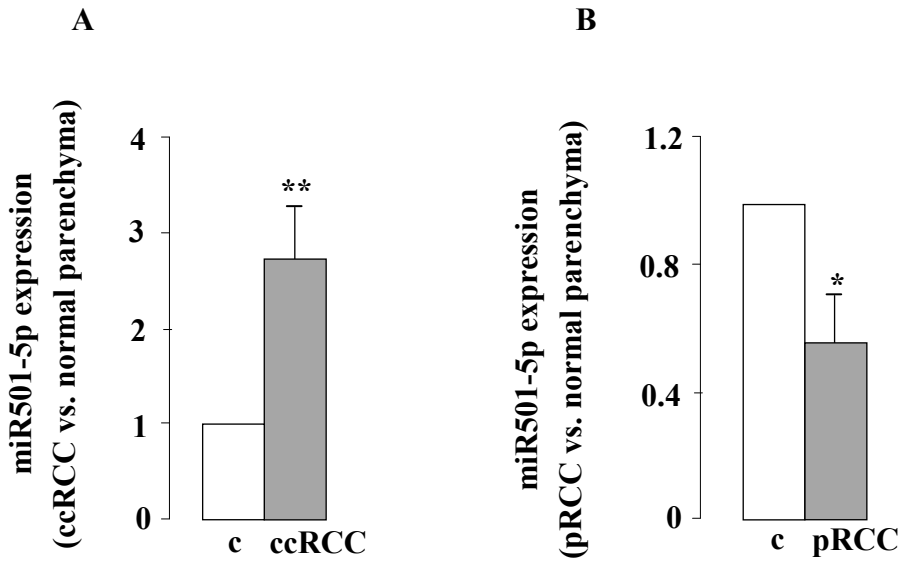


Figure 33: MicroRNA501-5p expression in kidney carcinomas.
Levels of miR501-5p were analysed by Real Time RT-PCR in 63 pairs of ccRCC and normal kidney tissues after RNA extraction. The same analysis was also conducted in 18 pairs of pRCC and normal kidney parenchyma. Samples derived from both fresh frozen and paraffin embedded tissues were collected in province of Ferrara (Northern Italy). Real Time RT-PCR data, expressed as ratio between cancer tissues and normal kidney parenchyma, were calculated by delta delta Ct method. In ccRCC tissues the expression of miR501-5p was 2.76 ± 0.61 fold increase compared with control tissues (**p<0.01). MicroRNA501-5p expression in pRCC tissues was 0.56 ± 0.2 fold with respect to normal tissue (*p<0.05). Statistical analysis was performed by t-test in three different experiments in duplicate. Value as indicates as mean ± standard error. C= control; ccRCC= clear cell renal cell carcinoma; pRCC= papillary renal cell carcinoma.

As previously described, the overall expression of miR501-5p in clear cell renal carcinoma was higher compared with normal tissues, however several ccRCC tissues did not show an higher expression of this miR as compared with normal parenchyma. In fact, as shown in Figure 14A, the expression values of miR501-5p in patients with ccRCC is extremely variable and did not suggest any correlation with the age of patient. Moreover, no relation with miR501-5p expression and tumor grading was observed (Figure 14B). As shown in the figure 14A, expression levels of miR501-5p is distributed mainly in three groups: one where the expression of tumor tissue is lower than the normal tissue; the second where the expression is comparable to normal parenchyma and, the last where the expression is greater than control. However, the main cluster showed an expression level similar to normal parenchyma (Figure 14A).

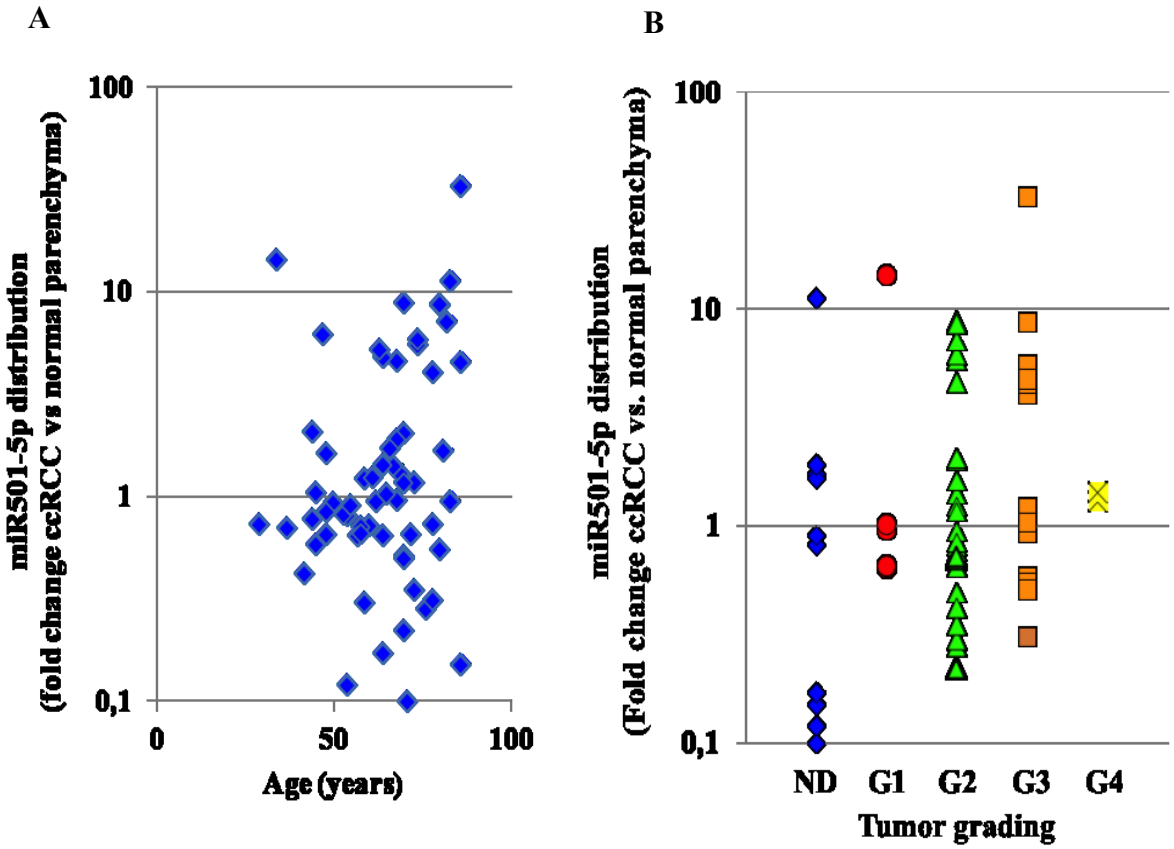


Figure 14: Expression values of miR501-5p related to the age of patients (A) and tumor grading (B).
Expression valued are expressed in logarithmic scale. No association with miR501-5p expression with patient's age or tumor grading was shown. ND= not determined, G= tumor grading (Fuhrman scale).

Because miR501-5p expression in clear cell renal carcinoma is extremely variable, we investigated whether this variability could affect the prognosis of patients with kidney carcinoma. Thus, we analysed the follow-up (at least five years) of 35 ccRCC and 9 pRCC subjects. For this analysis, we considered several parameters such as age of patients, histopathological features (Fuhrman grading), occurrence of metastatic, organ involved and patient survival. These parameters were correlated with the expression of miR501-5p (Table 6). As shown in the Table, the high expression of miR501-5p (marked in red) did not match with tumor grading or development of metastasis and, ccRCC patients with high or unchanged expression of this miR may exhibit a good as well as a poor prognosis. However, ccRCC samples with a low expression of miR501-5p (<0.8 versus normal parenchyma, values marked in blue) showed a good prognosis. These data were also confirmed in pRCC samples which showed a low expression of this MicroRNA (bottom of Table 6). These findings suggest that the reduced expression of miR501-5p seems to have a protective effect leading to a positive evolution of the disease in particularly in clear cell renal carcinoma.

Samples	Age	Grading	Metastasis	miR501	Therapy	Outcome
ccRCC1	86	ND	no	**0,15**	no	alive
ccRCC2	70	G2	no	**0,22**	no	alive
ccRCC3	76	G2	no	**0,28**	no	alive
ccRCC4	59	G2	no	**0,3**	no	alive
ccRCC5	78	G3	no	**0,31**	no	alive
ccRCC6	73	G2	no	**0,35**	no	alive
ccRCC7	70	G3	no	**0,51**	no	alive
ccRCC8	45	G3	no	**0,58**	no	alive
ccRCC9	54	G2	no	**0,81**	no	alive
ccRCC10	53	ND	no	**0,82**	no	alive
ccRCC11	50	G3	brain/lung	0,93	Anti VEGFR	dead
ccRCC12	83	G2	lung	0,95	Anti VEGFR	alive
ccRCC13	73	G3	no	1,17	no	alive
ccRCC14	59	G3	kidney	1,22	ND	ND
ccRCC15	61	G2	no	1,24	no	alive
ccRCC16	69	G4	lung/liver	1,3	ND	ND
ccRCC17	67	G2	no	1,41	no	alive
ccRCC18	64	G4	no	1,43	no	alive
ccRCC19	48	G2	no	1,62	no	alive
ccRCC20	70	G2	no	**2,03**	no	alive
ccRCC21	44	G2	no	**2,06**	no	alive
ccRCC22	78	G3	no	**4,06**	no	alive
ccRCC23	86	G3	larynx/urothelium	**4,53**	ND	ND
ccRCC24	68	G2	bones	**4,58**	ND	alive
ccRCC25	64	G3	brain/liver/lung	**4,77**	Anti VEGFR	dead
ccRCC26	63	G3	no	**5,24**	no	alive
ccRCC27	74	G3	no	**5,49**	no	alive
ccRCC28	74	G2	no	**5,86**	no	alive
ccRCC29	47	G2	lung/bones	**6,16**	Anti VEGFR	ND
ccRCC30	82	G2	lymph nodes	**7,17**	no	alive
ccRCC31	80	G2	no	**8,59**	no	alive
ccRCC32	80	G3	lung/bones	**8,7**	Anti VEGFR	dead
ccRCC33	70	G2	no	**8,82**	no	alive
ccRCC34	34	G1	lung	**14,36**	ND	alive

ccRCC35	86	G3	bones	**32,81**	ND	ND

pRCC1	82	G2	no	**0,06**	no	alive
pRCC2	74	G2	no	**0,11**	no	alive
pRCC3	53	ND	no	**0,19**	no	alive
pRCC4	69	ND	no	**0,23**	no	alive
pRCC5	79	G2	no	**0,26**	no	alive
pRCC6	51	G2	no	**0,51**	no	alive
pRCC7	79	G2	no	**0,55**	no	alive
pRCC8	85	G3	no	**0,94**	no	alive
pRCC9	71	ND	no	**3,91**	no	alive

Table 6: Follow-up analysis showed a good prognosis of ccRCC patients which express low levels of miR501-5p. For follow-up studied were taken into account patients with at least five years from surgical resection. Data derived from 35 ccRCC and 9 pRCC patients. The expression of miR501-5p was matched with different biological parameters (see top of the table).

From follow-up data appear that the increased expression of miR501-5p may be considered a risk factor for the clear cell renal carcinoma, but alone this microRNA was not able to induce the development of metastasis. Moreover, a low expression of miR501-5p was associated with a good prognosis for patients with clear cell kidney carcinoma. Therefore, miR501-5p could promote cell survival that, when combined with other factors, could lead to increased tumor aggressiveness, while, if it is downregulated, could stimulate apoptosis favouring a positive development of the disease.

4.2 Molecular role of miR501-5p.

In order to evaluate this hypothesis and to verify the role of miR501-5p in renal carcinoma, we investigated the possible molecular targets of this microRNA by the TargetScanHuman 6.0 program. Different mRNA target for miR501-5p have been identified and those related to apoptosis and cell proliferation are summarised in Table 7. In particular, were identified several mRNA including TSC1, different caspases, and PTEN. TSC1, a member of mTOR inhibitor complex, acts as oncosopressor modulating the pathway of mTOR. TSC1 interacting with TSC2 forms a complex able to block the protein Rheb, the activator of mTOR, resulting in the inhibition of this pathway.
Caspases are cysteine proteases that play essential roles in apoptosis; are present as inactive pro-enzymes that are activated by proteolytic cleavage. GAS2 is a caspase-3 substrate that plays a role in regulating microfilament and cell shape changes during apoptosis. It may also modulate cell susceptibility to p53-dependent apoptosis by inhibiting calpain activity. PTEN acts as tumor suppressor able to negatively regulate AKT signalling pathway.

Gene	Name	Function	Biological Processes
TSC1	tuberous sclerosis 1	tumor suppressor	mTOR signalling
Casp1	apoptosis-related cysteine peptidase	caspase cascade	apoptosis
Casp2	apoptosis-related cysteine peptidase	caspase cascade	apoptosis
Casp8	apoptosis-related cysteine peptidase	caspase cascade	apoptosis
Fas	cell surface death receptor	TNF receptor superfamily	apoptosis
GAS2	growth arrest-specific 2	caspase-3 substrate	apoptosis
CARD16	caspase recruitment domain family, member 1	caspase activator	Apoptosis
BLID	BH3-like motif containing, cell death inducer	caspase activator	pro apoptotic
BCL2L11	BCL2-Like 11 (Apoptosis Facilitator)	Bcl-2 family proteins	pro apoptotic
ING3	inhibitor of growth family, member 3	tumor suppressor	apoptosis
MCU	mitochondrial calcium uniporter	calcium channel	apoptosis
PTEN	phosphatase and tensin	tumor suppressor	apoptosis
DCC	deleted in colorectal carcinoma	tumor suppressor	apoptosis
MTSS1	metastasis suppressor 1	tumor suppressor	cell migration inhibition
BRCA1	breast cancer 1, early onset	tumor suppressor	DNA damage repair
Rictor	rapamycin-insensitive companion of mTOR	Subunit of mTORC2	cell growth
MAPK6	mitogen-activated protein kinase 6	protein kinase	cell proliferation, differentiation and development
MAP2K1	mitogen-activated protein kinase kinase 1	protein kinase	cell proliferation, differentiation and development
JUN	jun proto-oncogene	oncogene	gene expression regulation, tumorigenesis
KRAS	kirsten rat sarcoma viral oncogene homolog	oncogene	tumorigenesis
NRAS	neuroblastoma RAS viral (v-ras) oncogene homolog	oncogene	tumorigenesis
MYB	v-myb avian myeloblastosis viral oncogene homolog	oncogene	tumorigenesis
EGFR	epidermal growth factor receptor	protein tyrosine kinase	gene expression regulation, cell proliferation

Table 7: Putative mRNA targets of miR501-5p.
In this table are shown only the targets involving in cell growth and apoptosis. Molecular targets have been identified by using TargetScanHuman 6.0 program.

4.3 Analysis of miR501-5p upregulation in kidney carcinoma cells (KJ29).

To study the effects of miR501-5p on its targets, we used a human renal carcinoma cell line named KJ29. We have observed, in basal conditions, that these cells express higher levels of microRNA501-5p than normal tubular renal cells called 4/5, available in our laboratory (data not shown). Moreover, KJ29 cells were isolated from a not papillary human renal carcinoma tissue (Barletta et al, 1995). Therefore, we suppose that these cells may be considered an appropriate model for studying the function of this microRNA.

In order to verify the role of miR501-5p in kidney cancer cells, KJ29 cells were transfected with two different amounts (0.75 and 1.5 µg/mL) of a plasmid expressing specific miR501-5p sequences (PL501), which also contained sequences for the green fluorescent protein (GFP). Transfection efficiency was monitored by a fluorescence microscope which allowed to display the number of cells stained with GFP. As shown in Figure 15A, the transfection occurred with both amounts of plasmid (0.75 and 1.5 µg/mL), but with the lower dose, the fluorescence was present in a more number of cells as compared to cells transfected with 1.5 µg/mL of PL501. Indeed, we have observed that the transfection with 1.5 µg/mL of this plasmid induced toxic effects with significant detachment of cells from plate. For this reason, in subsequent experiments, we transfected the cells with 0.75 µg/mL of PL501 plasmid.

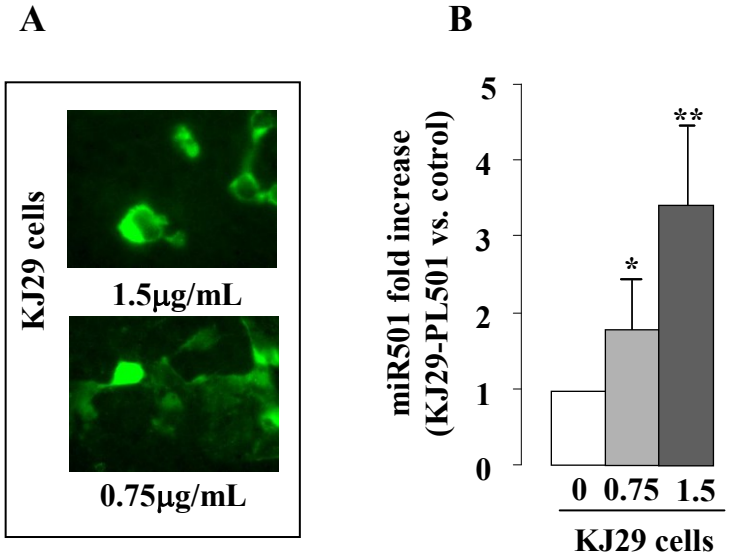


Figure 15:The transfection of KJ29 cells with PL501 increased the expression of miR501-5p in a dose dependent manner.

(A). Evaluation of transfection efficiency in KJ29 cells treated with 0.75 and 1.5 µg/mL of PL501 plasmid. Cells were seeded on glass coverslips, transfected for 24 h and analysed by a fluorescence microscope. Images were acquired through a CCD camera. (B) Analysis of miR501-5p expression by Real Time RT-PCR in KJ29 cells transfected for 24 h with the PL501 (0.75 and 1.5 µg/mL) or with an irrelevant plasmid (0). Data are expressed as fold increase calculated by the ratio among KJ29 cells transfected with PL501 and cells transfected with an irrelevant plasmid. Values were: 1.82± 0.6 and 3.4±1.2 for cells transfected with 0.75 and 1.5 µg/mL of PL501, respectively. Data are represented as mean ± standard deviation (SD) from three independent experiments (**$p<0.01$;*$p<0.05$).

To examine whether miR501-5p sequences were expressed after transfection, different amounts (0.75 and 1.5 µg/mL) of PL501 plasmid were used for cell transfection and miR501-5p expression was analysed by Real Time RT-PCR. As shown in the Figure 15B, the transfection of KJ29 cells with PL501 caused an increased expression of miR501-5p compared with cells transfected with an irrelevant plasmid. The increased expression of this miR after PL501 transfection occurred in a dose dependent manner.

4.4 MicroRNA501-5p upregulation promotes cell proliferation.

To investigate the possible impact of miR501-5p on cell proliferation, KJ29 cells were transfected for 24, 48 and 72 h with 0.75 µg/mL of PL501. Cell growth was evaluated by direct cell counting and with the CellTiter assay (for details, see method section). After 72 h of transfection, KJ29 cells exhibited a significant increase of cell proliferation compared with cells transfected with an irrelevant plasmid (Figure 16A). The treatment of KJ29 cells with 0.75 µg/mL of PL501 for 72 h also caused an increased cell survival, measured by CellTiter assay, as compared to the same cells transfected with an irrelevant plasmid (Figure 16B). These results indicated that miR501-5p upregulation stimulates cell proliferation as well as cell survival in KJ29 kidney carcinoma cells and could explain the development of metastasis observed in some samples with high levels of miR501-5p (Table 6).

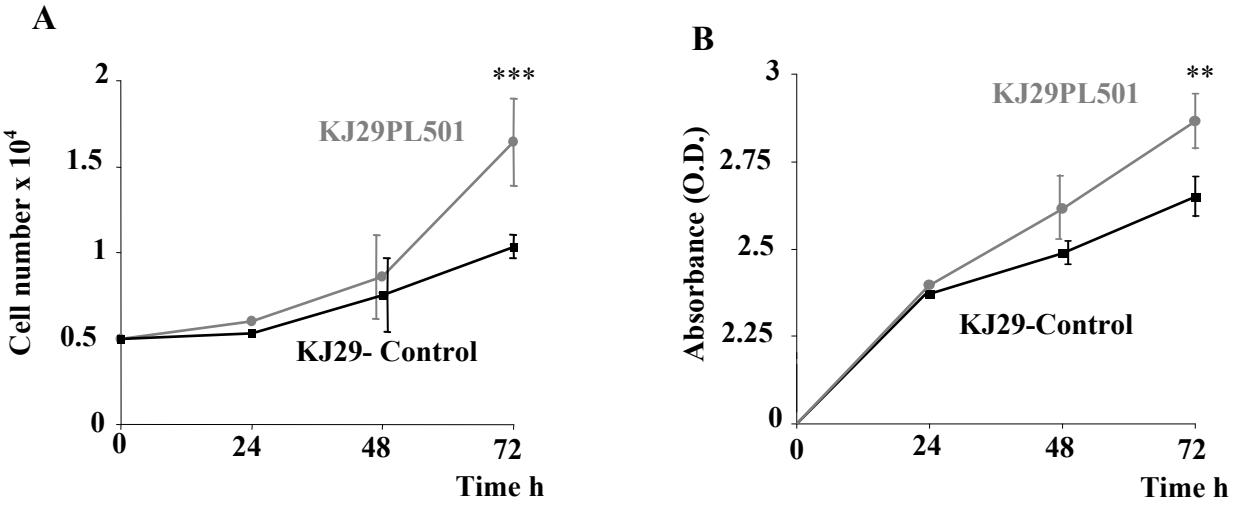


Figure 16: Analysis of cell proliferation and survival in KJ29 cell transfected with PL501.
(A). Analysis of cell proliferation by direct cell counting using a Burker chamber. Cells were transfected with 0.75 µg/mL of PL501 (KJ29-PL501) or with an irrelevant plasmid (KJ29-Control) and cultured in DMEM/F12 supplemented with 1% FBS for 24, 48 and 72 h. After 72 h of culture the number of cells transfected with PL501 was 32833±1917, while that of KJ29 cells transfected with an irrelevant plasmid was 20888±351.
(B) Analysis of cell survival by CellTiter assay. Cells transfected as described above were treated with formazan (see methods) and the absorbance was detected at 490 nM by a specific plate reader. After 72 h, the values of KJ29 treated with PL501 were 2.87±0.08 and those of control cells were 2.65±0.06. Data are represented as mean ± standard deviation (SD) from three independent experiments (**$p<0.01$; ***$p<0.001$).

4.5 MiR501-5p upregulation stimulates mTOR kinase activity.

As previously described, a target of miR501-5p is the mRNA for TSC1, a component of mTOR inhibitor complex. Therefore, the upregulation of this miR should reduce the expression of TSC1 activating the mTOR kinase. To verify this hypothesis KJ29 cells were transfected with the PL501 plasmid and the activity of mTOR was evaluated by western blot analysis. As expected, KJ29 cells transfected with PL501 showed an increased activity of mTOR compared with control cells (Figure 17). This data indicated that TSC1 mRNA is a real target for microRNA501-5p and, this miR is able to regulate the mTOR signalling in kidney carcinoma cells.

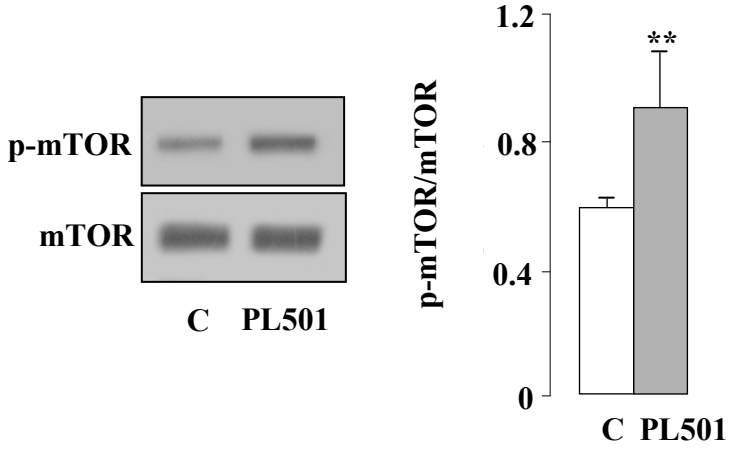


Figure 17 : Analysis of mTOR kinase activity in KJ29 cells overexpressing miR501-5p.
The activity of mTOR kinase was analysed by western blotting in KJ29 cells transfected for 24h with either PL501 and an irrelevant plasmid (C). Values calculates as ratio among the intensity of mTOR phosphorylated form and that of unphosphorylated band were: 0.58±0.019 for control cells and 0.89±0.17 for cells transfected with 0.75 μg/mL of PL501. Data are represented as mean ± standard deviation (SD) from three independent experiments (**p<0.01).

4.6 MiR501-5p, regulating the pathway of mTOR, modulates the expression of MDM2 and p53.

MDM2 protein (Murine Double Minute 2) functions as an ubiquitin E3 ligase to facilitate degradation of p53, a key regulator for cell proliferation, apoptosis and senescence in response to cellular stresses, such as DNA damage and oncogenic stress *(Bond et al, 2005)*. Whereas mTOR is able to positively regulate MDM2 through an increase in translation of MDM2 mRNA (*Moumen et al., 2007)*, we investigated levels of MDM2 and p53 proteins in cells overexpressing miR501-5p. As shown in Figure 18, KJ29 cells transfected with PL501 display increased levels of MDM2 protein and consequently a reduction of p53 protein compared with control cells. Therefore, the increased expression of miR501-5p degrading the mRNA of TSC1 should activate mTOR signalling which stimulating the expression of MDM2 leads to p53 ubiquitination and subsequent

protein degradation via proteasome. These events may promote apoptosis resistance ensuring an increased cell survival in particular in cells or tissues expressing high levels of miR501-5p.

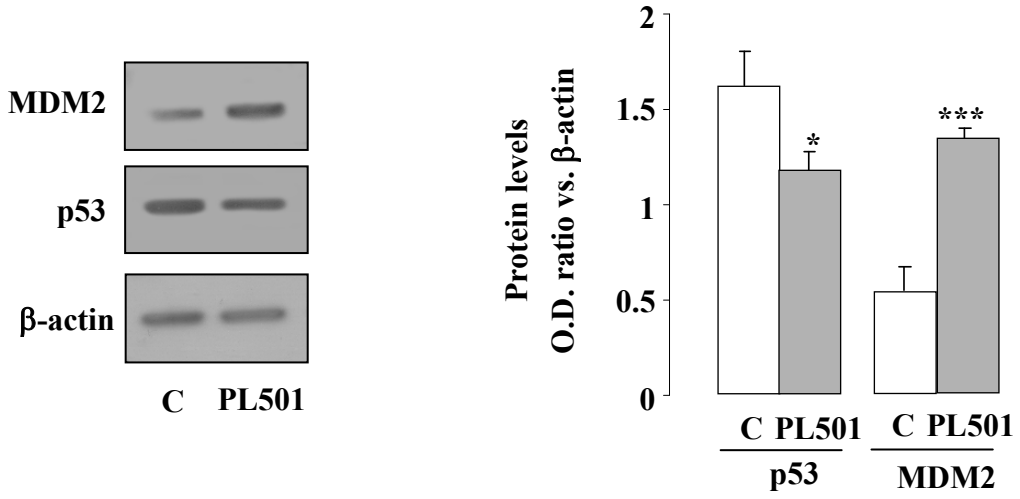


Figure 18: Western blot analysis of MDM2 and p53 in KJ29 cells transfected with PL501 plasmid.
Cells treated as described in Figure 16 were analysed to evaluate the expression of MDM2 and p53 proteins. Protein levels were calculated as ratio between the band of MDM2 or p53 and that corresponding to actin, used as housekeeping gene for the sample normalization. p53 protein levels were:1.62±0.17 in control cells and 1.17±0.10 in KJ29 cells transfected with PL501. Values for MDM2 protein were: 0.53±0.12 for control cells and 1.32±0.068 for PL501 transfected cells. Data are represented as mean ± standard deviation (SD) from three independent experiments (*p<0.05; ***p<0.001).

As previously reported, MDM2 causing p53 ubiquitination leads to protein degradation through the activation of proteasome in many cancer cells *(Bond et al, 2005)*. Therefore, the upregulation of miR501-5p stimulating the mTOR activity and MDM2 expression should induce p53 ubiquitination. Thus, KJ29 cells were transfected with PL501 and p53 polyubiquitination was evaluated by protein immunoprecipitation. As shown in Figure 19, a marked increase of protein ubiquitination in KJ29 cells transfected with PL501 compared with control cells was observed. This data may explain the reduction of p53 expression showed in cells overexpressing miR501-5p (Figures 18 and 19).

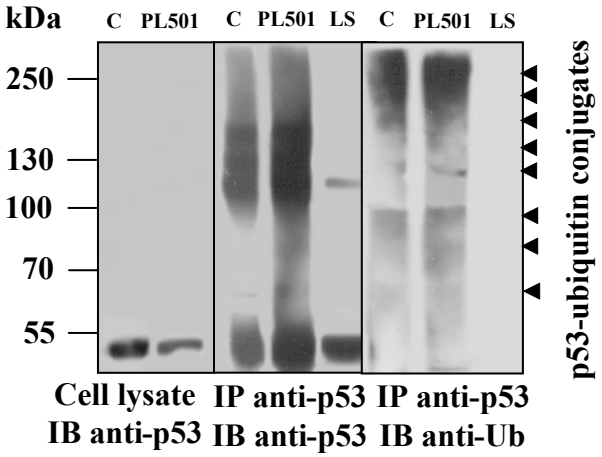


Figure 19: Analysis of p53 ubiquitination by western blotting in KJ29 cells overexpressing miR501-5p sequences.
After transfection, cells were treated with the proteasome inhibitor MG-132 (10 μM) for 4 h, lysates and an aliquot immunoprecipitated with an anti-p53 antibody. Subsequently, lysed and immunoprecipitated proteins were separated by electrophoresis and transferred on a nitrocellulose membrane. Finally, membranes were probed with anti-p53 and anti-ubiquitin antibodies and polyubiquitinated proteins were visualized by chemiluminescent assay (as described in method section). IB= immunoblot; IP= immunoprecipitation; C= protein lysate derived from cells transfected with an irrelevant plasmid; PL501= protein lysate from cells transfected with the plasmid expressing miR501-5p sequences; LS= lysis buffer .

The reduction of p53 expression in KJ29 cells which express high levels of miR501-5p occurs through the activation of mTOR kinase that in turn stimulates the expression of MDM2. Therefore, the inhibition of mTOR signalling should prevent the degradation of p53. To verify this assumption we treated KJ29 cells transfected with PL501 or with an irrelevant plasmid with rapamycin, an inhibitor of mTOR kinase and protein levels were analysed by western blot. As shown in Figure 20, in absence of rapamycin an increased activity of mTOR, a stimulation of MDM2 expression and a reduction of p53 protein levels in KJ29 cells transfected with 0.75 μg/mL of PL501 was observed. However, the application of rapamycin to the cells caused a marked reduction of both mTOR activity and MDM2 protein level with a concomitant increase in p53 expression (Figure 20). This last results is evident only in KJ29 cells transfected with PL501 which show an upregulation of miR501-5p as well as a strong mTOR kinase activity. These findings suggest that the rapamycin treatment and thus the inhibition of mTOR pathway is able to revert the p53 degradation associated with the upregulation of microRNA501-5p in kidney carcinoma cells.

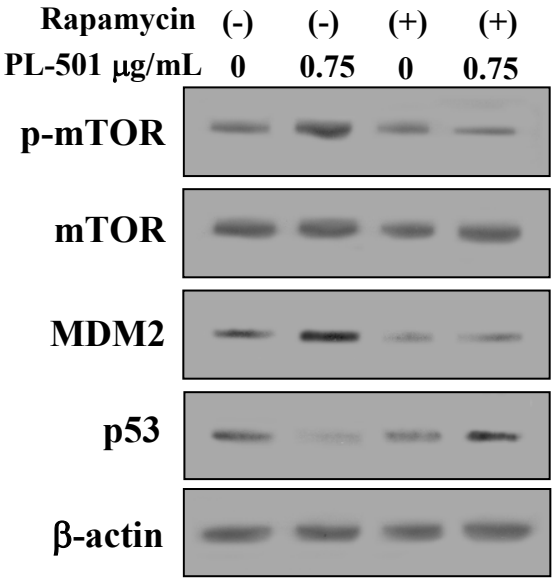


Figure 20: Western blot analysis of mTOR, MDM2 and p53 in cells transfected with PL501 and treated with rapamycin.
Cells were transfected with either PL501 (0.75 μg/mL) or irrelevant plasmid (0) for 24h and cultured for further 24 h in presence or absence of 500 nM rapamycin. Protein lysates were separated by electrophoresis and transferred on a nitrocellulose membrane. The different protein were probed with specific antibodies and visualized by a chemiluminescent assay.

We also investigated cell proliferation following administration of rapamycin because KJ29 cells which overexpressing miR501-5p shoved an increase in both cell proliferation and mTOR activity (Figures 16 and 17). Cell growth was observed in cells KJ29 transfected with an irrelevant plasmid and with PL501 (0,75 µg/mL). Figure 21A showed that cell proliferation, increased in KJ29 cells transfected with PL501 for 72 h, was reduced by the treatment of the same cells with rapamycin. Consistently, also cell survival was decreased after treatment with 500 nM rapamycin in KJ29 cells transfected with PL501 (Figure 21B). These data confirm that high levels of miR501-5p modulating the mTOR pathway stimulates cell proliferation in renal cancer cells. Thus, the treatment with rapamycin that induced a reduction of cell proliferation in KJ29 cells expressing high levels of miR501-5p, could improve the prognosis of patients with clear cell kidney carcinoma which show higher levels of both microRNA501-5p expression and mTOR activity.

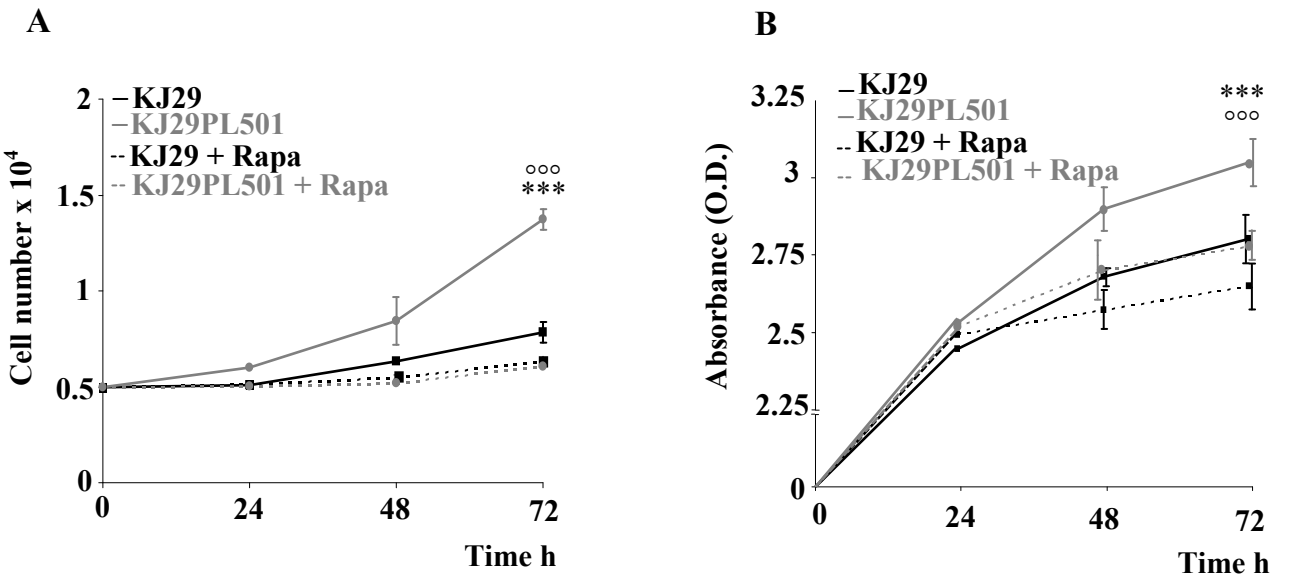


Figure 21: **Analysis of cell proliferation and survival in KJ29 cells transfected with PL501 and treated with rapamycin.**

(A). Analysis of cell proliferation by direct cell counting using a Burker chamber. Cells were transfected with 0.75 µg/mL of PL501 (KJ29-PL501) or with an irrelevant plasmid (KJ29-Control) and cultured in DMEM/F12 supplemented with 1% FBS in presence or absence of rapamycin (500 nM) for 24, 48 and 72 h. After 72 h of culture the number of cells transfected with PL501 was 13716±542, while that of KJ29 cells transfected with an irrelevant plasmid was 7822±534. (PL501 vs. control cells; ***$p<0.001$). The treatment with rapamycin caused a significant reduction of cell proliferation in KJ29 cells transfected with PL501. Values were 13716±542 for KJ29 cells transfected with PL501 and 6055±550 for KJ29 cells transfected with PL501 and treated with rapamycin. (PL501 untreated cells vs. PL501 cells treated with rapamycin; °°°$p<0.001$).

(B) Analysis of cell survival by CellTiter assay. Cells treated as described in point A were cultured in presence of formazan and then the absorbance was detected at 490 nM by a specific plate reader. After 72 h, values of KJ29 treated with PL501 were 3.04±0.078 and those of control cells were 2.80±0.076. (***$p<0.001$).

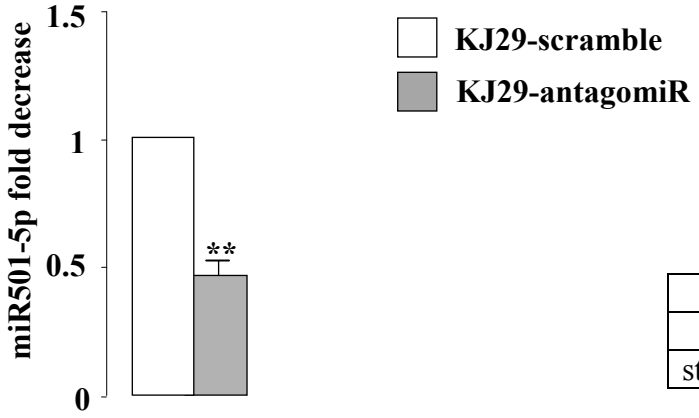
values of KJ29 cells transfected with PL501 treated with rapamycin were 2.77±0.04. (PL501 untreated cells vs. PL501 cells treated with rapamycin; °°°$p<0.001$)

Data are represented as mean ± standard deviation (SD) from three independent experiments in duplicate.

Taken together this findings suggest that miR501-5p upregulation could cause apoptosis resistance and promote cell survival. This hypothesis is consistent with the poor prognosis observed in some ccRCC patients. However, not all the patients which express high levels of miR501-5p develop metastasis, therefore the upregulation of this miR may be a risk factor that if associated with other events may cause a worse evolution of clear cell renal carcinoma.

4.7 MiR501-5p evaluation in KJ29 transfected with antagomiR

Since the low expression of miR501-5p is associated with a good prognosis for ccRCC patients, the downregulation of this miR could have a protective effects on these ccRCC patients. To test this hypothesis, we reduced expression of miR501-5p through a specific antagomiR. Cells KJ29 were transfected with antagomiR and after 24 h the expression of miR501-5p was evaluated by Real Rime RT-PCR. The values obtained in transfected cells are approximately 50% lower as compared to cells transfected with scramble sequences (Figure 22). This result suggests that the treatment with antagomiR is able to reduce the expression of miR501-5p in KJ29 kidney carcinoma cells.



	KJ29 (-)	KJ29 (+)
mean	1	0.47
standard deviation	/	0.03

Figure 42: Analysis miR501-5p expression by real time PCR in KJ29 transfected with antagomiR.
The analysis of miR501-5p expression was performed by Real Time RT-PCR in KJ29 cells transfected for 24 h with a specific antagomiR or with scramble sequences. Data are expressed as fold change among KJ29 cells transfected with antagomiR and cells transfected with scramble. Values were: 0.47± 0.03 for cells transfected with antagomiR. Data are represented as mean ± standard deviation (SD) from three independent experiments (**$p<0.01$).

To verify the effect of miR501-5p downregulation we treated KJ29 cells with a specific antagomiR and cell proliferation and survival was investigated by CellTiter assay. The analysis of cell

proliferation was conducted for 24h and 48h after transfection of KJ29 cells with antagomiR or with scramble sequences (control cells). The transfection with antagomiR for 48h caused a marked reduction of absorbance indicating a decrease of cell growth compared with control cells (Figure 23). Otherwise from miR501-5p upregulation, reduced levels of this miR caused a strong decrease of cell proliferation.

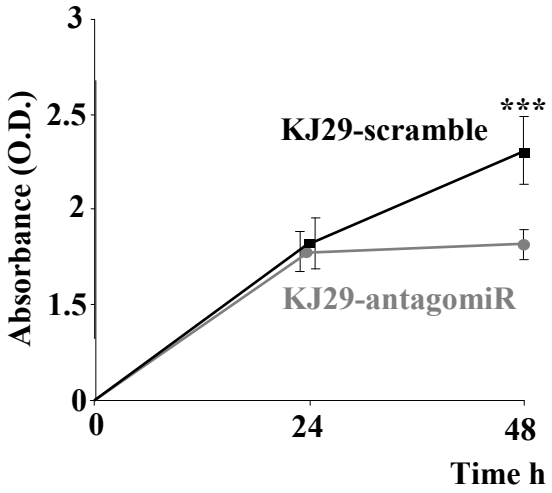


Figure 23: Analysis of cell growth by CellTiter assay in cell KJ29 transfected with antagomiR.
Cell were transfected for 24 and 48 h with antagomiR (30nM) or with scramble sequences. after transfection cell growth were measured by CellTiter assay, as previously reported. values of KJ29 treated with antagomiR were 1.81±0.077 and those of scramble cells were 2.31±0.17. (***$p<0.001$). Data are represented as mean ± standard deviation (SD) from three independent experiments.

4.8 Analysis of cell cycle and caspase-3 in KJ29 cells transfected with antagomiR.

To confirm the reduction of cell growth obtained by CellTiter assay, we have investigated cell proliferation also by cell cycle analysis in KJ29 cells transfected with either antagomiR and scramble sequences. As shown in Figure 24 an increased G0/G1 followed from a reduction of S phase in KJ29 cells treated with antagomiR compared with control cells was observed. These findings indicated that a low expression of miR501-5p induced a reduction of cell growth by cell accumulation in G0/G1 phase of cell cycle in renal carcinoma cells. Moreover, these data are consistent with the good prognosis observed in ccRCC patients which express lower levels of miR501-5p.

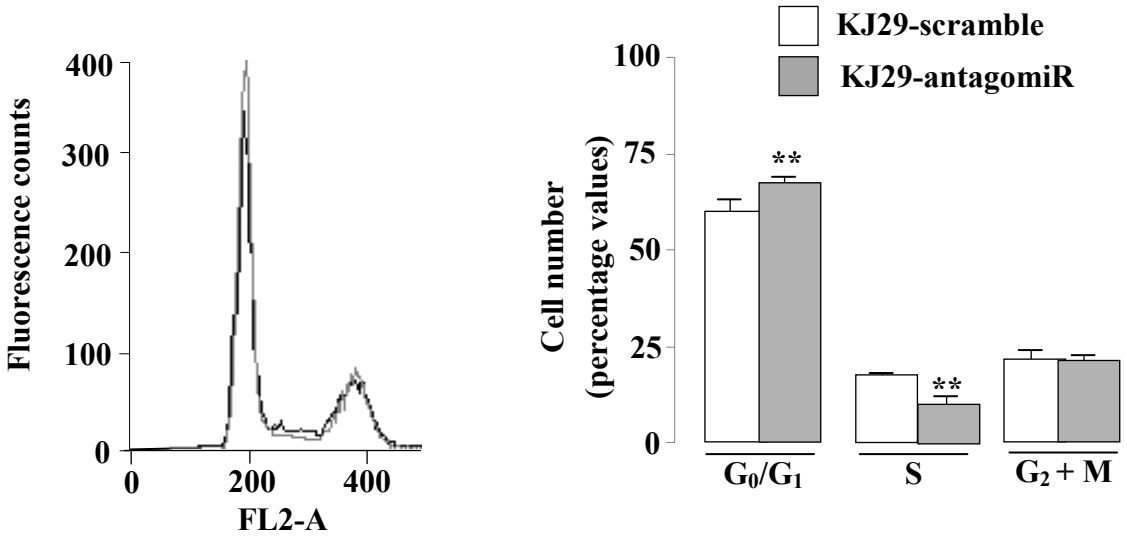


Figure 24: Analysis of cell cycle in KJ29 treated with antagomiR of with scramble sequences.
After transfection, cells were cultured for 24 h in DMEM/F12 supplemented with 0.4% BSA, resuspended in propidium iodide and analysed by flow cytometry. Cycle analysis showed a significant increase in G0/G1 phase in KJ29 cells treated with antagomiR (grey line) compared with control cells (dark line). Values for G0/G1 phase were 67.5±0.5% for KJ29 antagomiR and 60.5±0.7% for KJ29 scramble (**$p<0.01$). Values for S phase were 11±0.2% for KJ29 antagomiR and 17.5±0.1% for KJ29 scramble (**$p<0.01$). Data are expressed as mean ± standard deviation from three different experiments in duplicate.

As reported in Table 7, miR501-5p showed several mRNA targets including different caspases which are involved in the apoptotic machinery. Therefore, a reduction in miR501-5p sequences should enhance the caspase activity. In order to evaluate this assumption we have analysed caspase-3 activity, the last enzyme of apoptotic cascade, by a specific caspase-3 assay in KJ29 cells downregulated for microRNA501-5p. Figure 25 showed an evident increase in caspase-3 activity in KJ29 cells treated with antagomiR compared with those treated with scramble sequences. As expected from targets of this miR, its decreased expression caused a significant increasing of caspase activity suggesting that low levels of this miR may activate cellular apoptosis and, thus showing a protective effect for ccRCC patients that express few sequences of microRNA501-5p. To confirm that the downregulation of miR501-5p may activate apoptosis, we have analysed the presence of apoptotic nuclei in KJ29 cells treated with antagomiR by Hoechst method. As shown in Figure 26, the reduction of miR501-5p expression caused the formation of apoptotic nuclei not observed in KJ29 control cells. This result further supports a protective effect of miR501-5p downregulation that occurs by the activation of apoptosis in renal cancer cells.

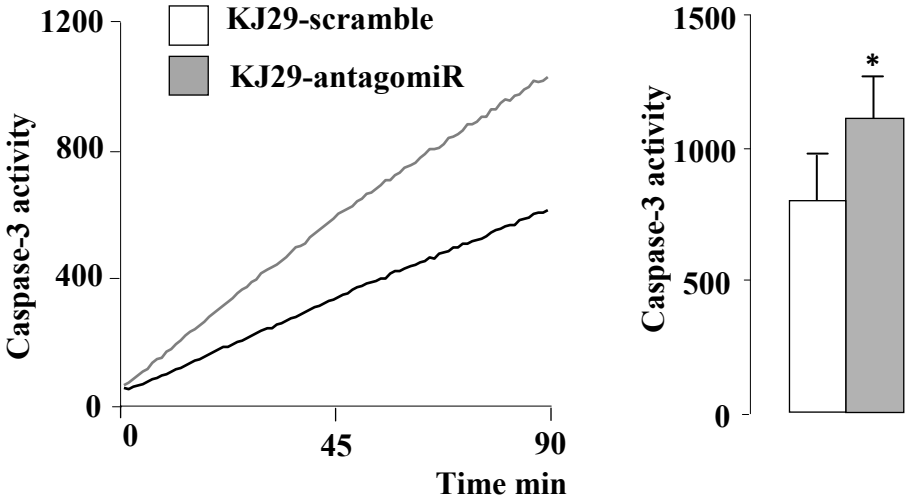


Figure 25: Evaluation of caspase-3 activity in KJ29 cells treated with antagomiR or with scramble sequences. KJ29 cells were transfected with either antagomiR and scramble sequences in DMEM/F12 medium plus 0.4% BSA for 24h. Then, cells were collected and analysed for caspase-3 activity as reported in method section. Values for KJ29 antagomiR were: 1027±155.3 (fluorescence units), while those of KJ29 scramble were 612±40.7. (*$p<0.05$). Data are expressed as mean ± standard deviation from three different experiments in duplicate.

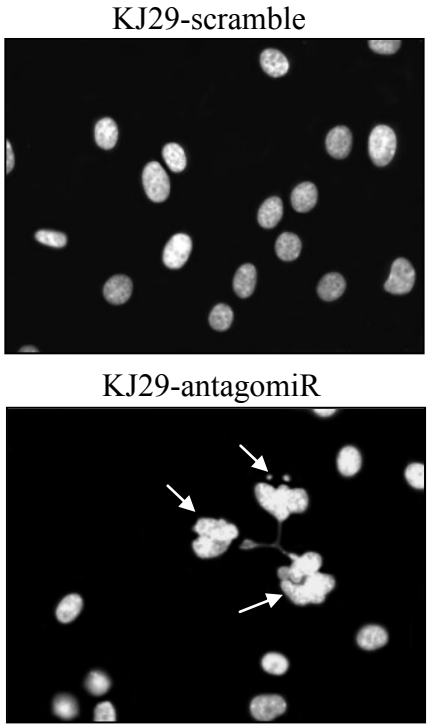


Figure 26: Analysis of apoptosis by Hoechst method after downregulation of miR501-5p in KJ29 cells.
KJ29 cells, seeded on coverslips, were transfected with either antagomiR and scramble sequences in DMEM/F12 medium plus 0.4% BSA for 24h. Then, cells were fixed, permeabilized and stained with Hoechst solution. Images were collected by a fluorescence microscope equipped with a CCD camera. Pictures were recorded at 50x magnification. Arrows indicate apoptotic nuclei.

4.9 Western blot analysis of mTOR, p53 and MDM2 in KJ29 transfected with antagomiR.

As previously reported miR501-5p upregulation promoted mTOR activity in KJ29 cells likely by degradation of TSC1 mRNA which is a target of this miR as well as an inhibitor component of mTOR kinase. Therefore, a reduction of microRNA501-5p should preserve TSC1 mRNA and cause a reduction of mTOR activity. In fact, the dowregulation of this miR by application of antagomiR to KJ29 cells induced a marked reduction of mTOR protein phosphorylation compared with cells transfected with scramble sequences (Figure 27). This clearly indicates that the reduction of mTOR activity is closely associated with the decreasing of miR501-5p expression and this would confirm an important role of this miR in the modulation of mTOR signaling.

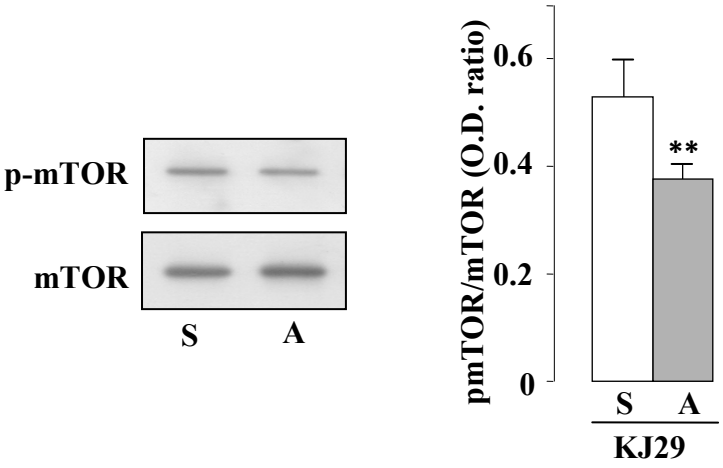


Figure 27: Analysis of mTOR kinase activity in KJ29 cells with reduced levels of miR501-5p.
The activity of mTOR kinase was analysed by western blotting in KJ29 cells transfected for 24h with either antagomiR (A) and scramble sequences (S). Values calculates as ratio among the intensity of mTOR phosphorylated form and that of unphosphorylated band were: 0.52±0.06 for control cells and 0.37±0.03 for cells transfected with antagomiR. Data are represented as mean ± standard deviation (SD) from three independent experiments (**$p<0.01$).

It is known that mTOR signalling is able to stimulate MDM2 expression (*Moumen et al., 2007)* therefore, a reduction of mTOR activity should cause a decreasing in MDM2 expression. In fact, in KJ29 cells treated with antagomiR which exhibit a reduction of both miR501-5p expression and mTOR activity, a lower expression of MDM2 protein compared with control cells was observed (Figure 28). This finding showed that the reduction of miR501-5p induced a downregulation of MDM2 protein via negative regulation of mTOR pathway in kidney cancer cells. Moreover, the downregulation of this miR caused opposite effects with respect to its upregulation and thus would confirm the bona fide of our data.

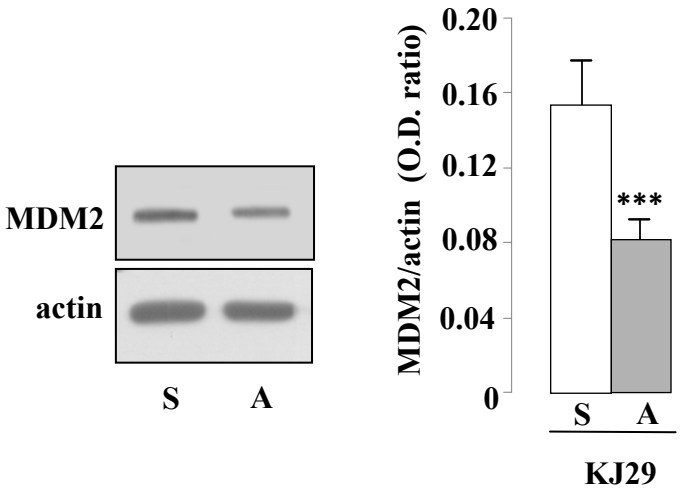


Figure 28: Western blot analysis of MDM2 in KJ29 cells transfected with antagomiR.
Cells transfected with antagomiR (A) or scramble sequences (S) were analysed to evaluate the expression of MDM2 protein. Protein levels were calculated as ratio between the band of MDM2 and that corresponding to actin, used as housekeeping gene for the sample normalization. Values for MDM2 protein were: 0.15±0.02 for control cells and 0.08±0.01 for antagomiR transfected cells. Data are represented as mean ± standard deviation (SD) from three independent experiments (***p<0.001).

Apoptotic processes are often associated with activation of p53 protein (*Polyak K. et al, 1997*), which regulates the cell cycle and stimulates apoptosis, acting as a tumor suppressor. Moreover, the expression of p53 may be inhibited by MDM2 protein. Because we have shown that miR501-5p downregulation caused a reduction of MDM2 expression (Figure 28) it is reasonable to think that reduced levels of MDM2 protein may be associated with an increased expression of p53. Consistently, the treatment of KJ29 cells with antagomiR stimulated the expression of p53 protein as compared to control cells (Figure 29). These results indicate that the downregulation of microRNA501-5p could have an anti-apoptotic effect that occurs by the reduction of MDM2 expression and the decrease of mTOR kinase activity.

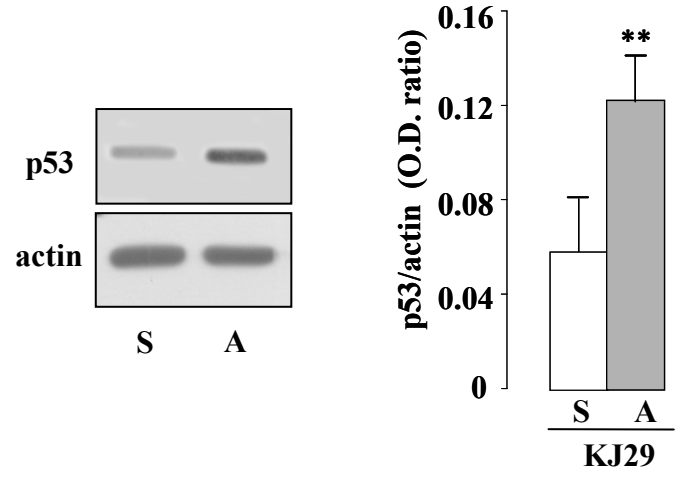


Figure 29: Western blot analysis of p53 in KJ29 cells transfected with antagomiR.
Cells transfected with antagomir (A) or scramble sequences (S) were analysed to evaluate the expression of p53 protein. Protein levels were calculated as ratio between the band of p53 and that corresponding to actin, used as housekeeping

gene for the sample normalization. Values of p53 protein were: 0.58±0.03 for control cells and 0.123±0.018 for antagomiR transfected cells. Data are represented as mean ± standard deviation (SD) from three independent experiments (**p<0.01).

To functionally evaluate the increased expression of p53 in KJ29 cells treated with antagomiR, we have performed experiments of protein nuclear translocation using a fluorescence microscope. Cells were treated with specific anti-p53 primary antibodies and stained by application of a secondary antibody conjugated with rhodamine. As shown in figure 30, a marked p53 protein nuclear translocation in KJ29 cells transfected with antagomiR was observed, while the fluorescence remained mainly confined to the cytoplasm in control cells. These data confirmed those observed by western blotting analysis and indicated that the reduced miR501-5p expression induced not only an increased expression of p53 but also an higher protein activation in kidney cancer cells.

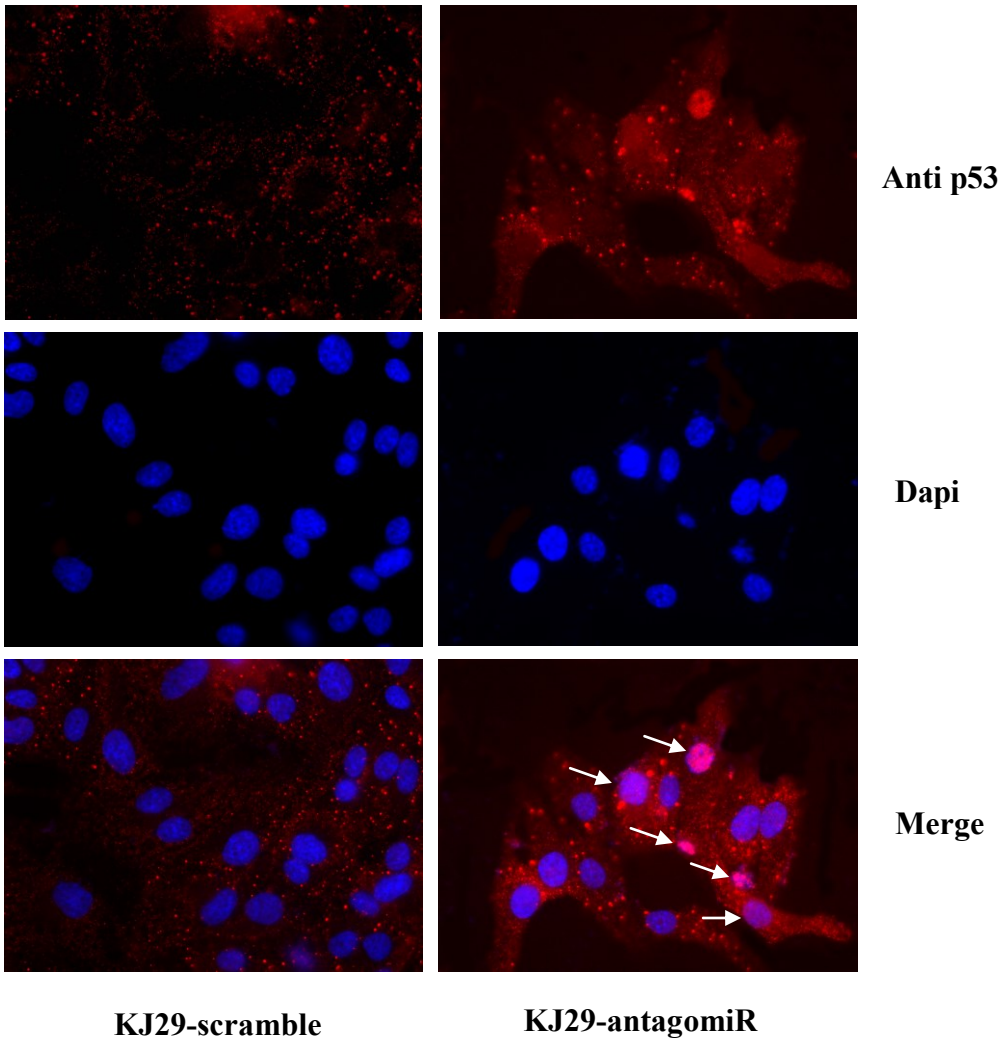


Figure 30: Analysis of p53 nuclear translocation by immunofluorescence in KJ29 cells transfected with antagomiR.
After transfection occurred in DMEM/F12 supplemented with 0.4% BSA on specific coverslips, cells were fixed, permeabilized and treated with an anti-p53 monoclonal antibody. After several washes cells were treated with a secondary antibody conjugated with rhodamine. The staining of nuclei was performed with Dapi and images were

acquired with a 40x magnification by a fluorescence microscope equipped with a CCD camera. Arrows show the immunopositive nuclei.

Taken together these results suggest that miR501-5p downregulation promoted the accumulation of cells in G0/G1 phase of cell cycle, caspase-3 activation and apoptotic nuclei formation. These events occurred by an increased expression and function of p53 through the inhibition of mTOR signalling that led to the reduction of MDM2 expression.

4.10 Expression of p53 and MDM2 in ccRCC tissues.

Our data showed that the downregulation of miR501-5p stimulated cell cycle accumulation in G0/G1 phase of cell cycle and promoted apoptosis in renal carcinoma cells. These finding are consistent with the good evolution of the disease observed in ccRCC patients which express low levels of this miR (Table 6). To confirm data observed in kidney carcinoma cells also in cancer tissues derived from ccRCC patients, we have analysed the expression of MDM2 and p53 proteins in ccRCC tissues with different expression of miR501-5p and in the corresponding normal renal parenchyma. As shown in Figure 31, the samples with lower levels of miR501-5p (red box) showed a reduction of MDM2 protein and an increased level of p53 compared with normal tissue (N). Conversely, tissues with unchanged of higher levels of miR501-5p did not show significant changes in MDM2 expression levels, while the expression of p53 appeared lower in ccRCC tissues than in normal parenchyma (Figure 31).

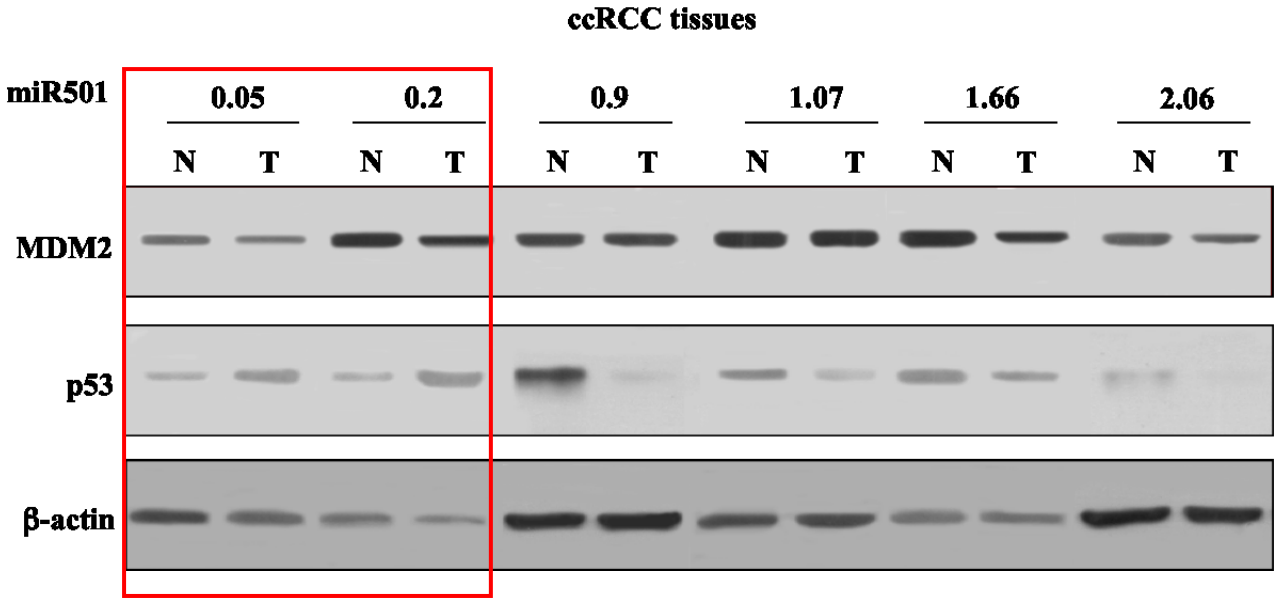


Figure 51: Analysis of p53 and MDM2 expression by western blotting in clear cell renal carcinoma tissues (ccRCC) and in normal renal parenchyma.
Normal kidney (N) and ccRCC tissues (T) were provided from the Urology Unit of St. Anna hospital after surgical resection. MicroRNA501-5p expression was evaluated by Real Time RT-PCR, while the protein levels of MDM2 and

p53 was analysed by western blotting. Sample normalization was done comparing Mdm2 and p53 bands with the protein band of β-actin, used as housekeeping gene.

This finding showed an association between data found in KJ29 cells that express low levels of miR501-5p and those observed in cancer tissues derived from patients which showed a downregulation of this microRNA. These results suggest that the downregulation of miR501-5p could play an pro-apoptotic role stimulating the expression of p53 and inducing an increase of caspase-3 activity. This should limit cell survival favouring a good prognosis in ccRCC patients, therefore miR501-5p expression may be used as a possible biomarker for the prognosis of clear cell kidney carcinoma.

5. CONCLUSIONS

Our results showed that the upregulation of miR501-5p could play an anti-apoptotic role stimulating cell growth and survival by the activation of mTOR signaling that in turn caused the increased expression of MDM2 protein and a reduction of p53 protein levels. Consistently, recent studies (*Yamamoto et al., 2009*) reported that the microRNA-500, belonging to the same cluster of miR501, may be used as potential diagnostic marker for hepatocellular carcinoma. In fact, miR-500 was abundantly expressed in several human liver cancer cell lines and 45% of human hepatocellular carcinoma (HCC) tissues. Moreover, this miR showed oncofetal properties and was aberrantly expressed in hepatocellular carcinoma. On the other hand, the downregulation of miR501-5p was able to promote apoptosis by activation of caspase-3 and the accumulation of cells in G0/G1 phase in a mechanism involving the activation of p53. Thus, lower levels of this miR showed a protective effect against the development of metastasis, in fact, ccRCC patients which express few sequences of microRNA501-5p showed a good prognosis compared with patients with unchanged or higher levers of this miR (Table 6).

As shown in Figure 31, miR501-5p could modulate the expression/activity of several proteins directly involved in cell growth, survival and apoptosis. Therefore, it could act as tip the balance between apoptosis and cell survival. When it is downregulated stimulates apoptosis through activation of caspase-3 and p53 protein. Conversely, when it is upregulated should act as stimulator of cell survival through activation of the mTOR pathway and the inhibition of p53. This mechanism may explain the favorable prognosis of patients with low levels of miR501-5p.

Finally, miR501-5p expression might be considered as a prognostic biomarker for the clear cell renal cell carcinoma and could be useful for the identification of treatable ccRCC patients.

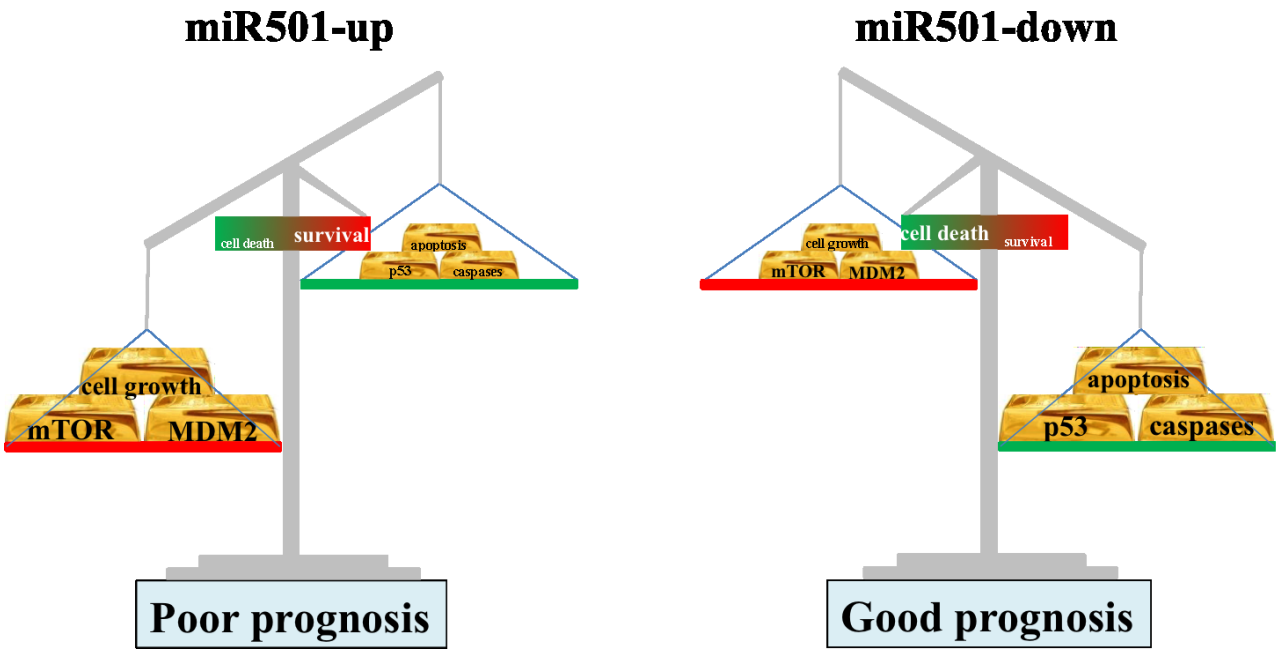


Figure 31: Schematic picture showing the possible role of microRNA501-5p.
The high expression of miR501-5p stimulates cell proliferation by increase of mTOR activity and MDM2 expression with consequent poor prognosis for ccRCC patients. While the down regulation of this miR leads to an increased apoptosis through activation of caspase-3 and p53 protein.

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