miRNAs: Redux

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Micro RNAs have become a significant element in diagnostic and prognostic efforts on a variety of cancers. We examine some of the current issues herein and discuss methods of using miRNAs as both diagnostic and prognostic tools.

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1 INTRODUCTION

Micro RNAs have been known for about thirty years. During the last ten years there has been an explosion in the discovery, identification, and operation of these short stretches of single stranded RNAs. These small segments of RNA can act in powerful ways to silence gene expression by binding to mRNAs before they can effect actionable proteins or cutting them apart. This note is an attempt to examine the current status of these significant factor in cellular functioning and especially in cancer initiation, control and metastasis.

In early 2019 the paper by Alles et al noted:

While the number of human miRNA candidates continuously increases, only a few of them are completely characterized and experimentally validated. Toward determining the total number of true miRNAs, we employed a combined in silico high- and experimental low-throughput validation strategy.

We collected 28 866 human small RNA sequencing data sets containing 363.7 billion sequencing reads and excluded falsely annotated and low-quality data. Our high-throughput analysis identified 65% of 24 127 mature miRNA candidates as likely false-positives. Using northern blotting, we experimentally validated miRBase entries and novel miRNA candidates.

By exogenous overexpression of 108 precursors that encode 205 mature miRNAs, we confirmed 68.5% of the miRBase entries with the confirmation rate going up to 94.4% for the high-confidence entries and 18.3% of the novel miRNA candidates. Analyzing endogenous miRNAs, we verified the expression of 8 miRNAs in 12 different human cell lines.

In total, we extrapolated 2300 true human mature miRNAs, 1115 of which are currently annotated in miRBase V22. *The experimentally validated miRNAs will contribute to revising targetomes hypothesized by utilizing falsely annotated miRNAs.*

There are still many unanswered questions regarding miRNAs. For example:

1. What initiates the generation of an miRNA. The 2300 or so miRNAs are present in some cells and not others, and why.

2. What are the promoters and initiators of these miRNA transcriptions. Are there upstream genetic characteristics and elements that make for their transcription.

3. miRNAs can inhibit transcribed mRNA thus inhibiting translation. What is the overall chemical reactivity of each which dominates. For example, if a cell can produce 1 miRNA per day but it can produce 2 mRNA per day, perhaps only half are blocked and there would be a de minimis effect. On the otherhand if the miRNA can survive the mRNA degradation then it has a perpetual existence. The reaction dynamics of this overall process is yet to be understood.

4. In cancers, we have identified a set of miRNAs, many of which are common across a large set of malignancies. The question is; is the driver of the malignant process the miRNA or some upstream genetic action?

5. Why do some cells activate miRNAs and others do not?

6. Can we develop upstream and downstream maps for any miRNA?

7. Is there a stochiometric system for the analysis of miRNA interactions?

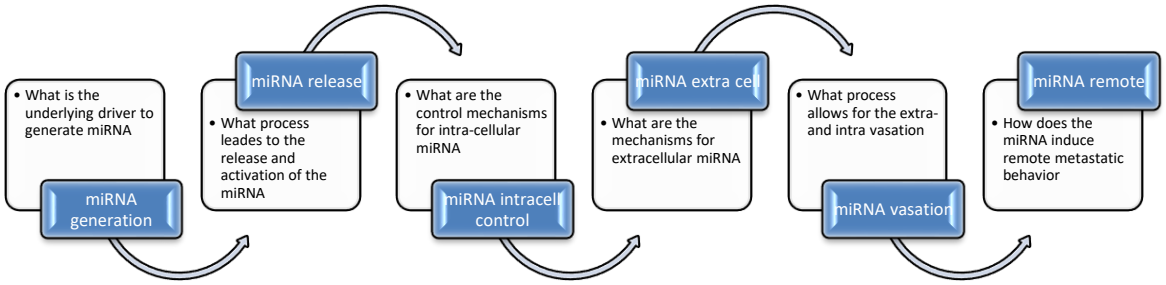
8. As miRNAs flow from the nucleus (as works in progress), what are the detailed steps by which they may be mitigated against.

9. The relationships between the up and down stream elements and the related miRNAs have a dynamic characteristic where concentrations of each entity in such a chain could be characterized. Can this be ascertained and demonstrated?

10. Are miRNA, as well as up and down stream entities targetable by therapeutics and if so how.

11. Targeting as above, what are the "unintended consequences" across homeostatic processes?

12. miRNAs via exosomes or even directly can be effectors of metastasis. How is this accomplished and how can it be mitigated?



In this note our objective is to overview the miRNA status, examine the miRNAs for a set of malignancies, overview the methodology to measure these miRNAs and then to explore the options to link the measurements to diagnostic and prognostic measures.

The challenges we see facing this effort clinically are:

1. Identification of miRNAs and malignant states

2. Measurement of the miRNAs in an effective and reasonable cot manner

3. The ability to identify a specific lesion despite the prolific presence of miRNAs across many possible ones.

4. The development of a metric which maps the miRNA measures into diagnostic and prognostic results by lesion type.

We examine the information currently available and consider a set of classifiers which could be employed. The challenges are that many of the input data elements are still works in progress and that unlike many current approaches such as PSA, which has its own limitations, we may not yet understand the miRNA metric limitations.

2 MIRNAS

We present a brief summary of miRNAs, their generation, and the basic principles related to cancers. The understanding of miRNAs is a continuing process and in reviewing the literature the initial understanding starting in 1993 was limited but it has recently exploded in detail. One of the earliest was the let or lethal miRNA, known as let-7. As Su et al have noted:

MicroRNAs (miRNAs) are a group of small noncoding RNAs capable of regulating specific gene expression. Let-7 miRNA was first discovered in Caenorhabditis elegans and it is highly conserved in human tissues. The human let- 7 family of miRNA contains 12 members of miRNA. Today, these members have become the most studied miRNAs and they have attracted attention of researchers in various fields, such as development, stem cell biology, aging, and metabolism.

Furthermore, there is a large body of evidence linking the loss of let-7 expression and the development of poorly differentiated, aggressive cancers. In addition to the canonical biogenesis pathway, let-7 has been found to be regulated by protein factors, such as RNA binding proteins previously identified as regulators of protein-coding mRNAs. Moreover, the direct interaction between miRNAs has recently been identified as a novel pathway to control let-7 expression. In this review, we discuss the multifaceted roles of let-7 and provide an overview of its regulation at multiple levels.

Su et al provide an excellent summary of how much detail is known about let-7. Specifically, we know how it is initiated and generated and how it controls downstream elements. let-7 provides a useful baseline for examining the 2300 or so miRNAs.

2.1 GENERATION

In the classic review paper by Esquela-Kerscher and Slack, they present an excellent discussion on miRNAs. First, we present the overall construct. miRNAs are produced like all RNA and then pass through the Drosha/Pasha complex and emerge from the nucleus as a double RNA with a loop. Dicer cuts the loop creating single strand short RNAs which are the miRNA.

The generation of miRNAs follow several possible pathways. Yang and Li had outlined some of these previously. The simplest explanation is that of the canonical pathway which we explain below.

1. The canonical pathway starts with the reading of a gene section with a Polymerase II (Pol II) and the production of a pri-miRNA (primary miRNA) molecule. This is a complex nucleotide molecule with loops and a poly A tail.

2. Then the enzyme Drosha, along with co-factor Pasha in the nucleus, cut the tails and the pre-miRNA is formed which is smaller but still with loops about 70 nt long. This sequence has a hairpin segment.

3. Exportin moves the duplex molecule outside the nucleus into the cytoplasm.

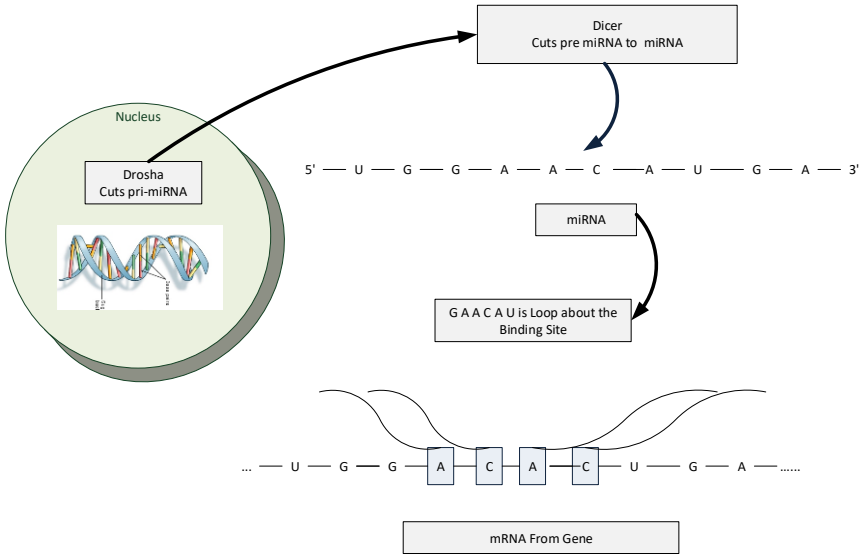
4. In the cytoplasm, Dicer cuts the pre-miRNA into two sections, the one without the loop being the mature miRNA.

5. At that point the mature miRNA finds a mature mRNA to which it binds to a complement site and at that point the mRNA is inactivated.

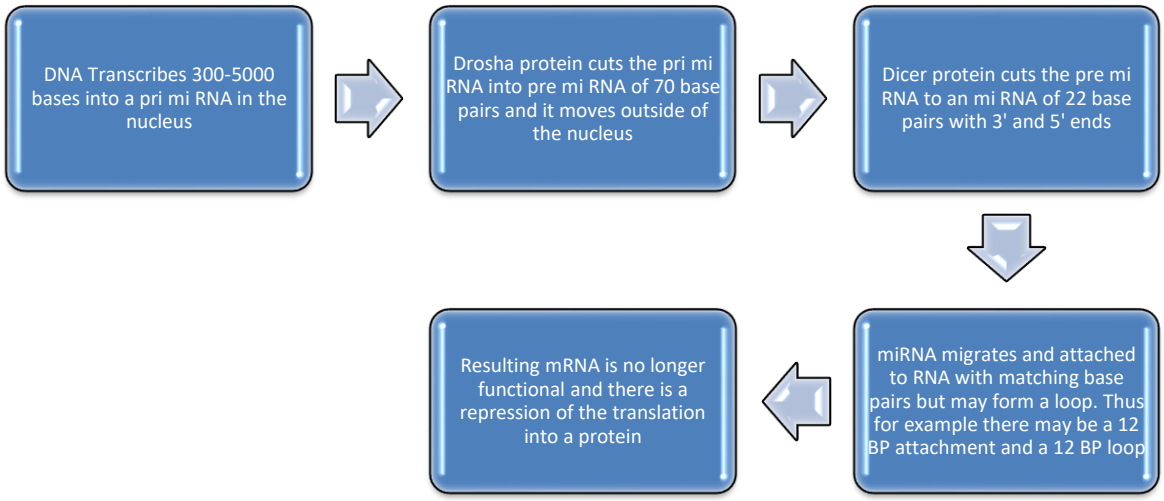
6. Specifically as Esquela-Kerscher and Slack had noted:

The mature miRNA then binds to complementary sites in the mRNA target to negatively regulate gene expression in one of two ways that depend on the degree of complementarity between the miRNA and its target. miRNAs that bind to mRNA targets with imperfect complementarity block target gene expression at the level of protein translation. However, recent evidence indicates that miRNAs might also affect mRNA stability.

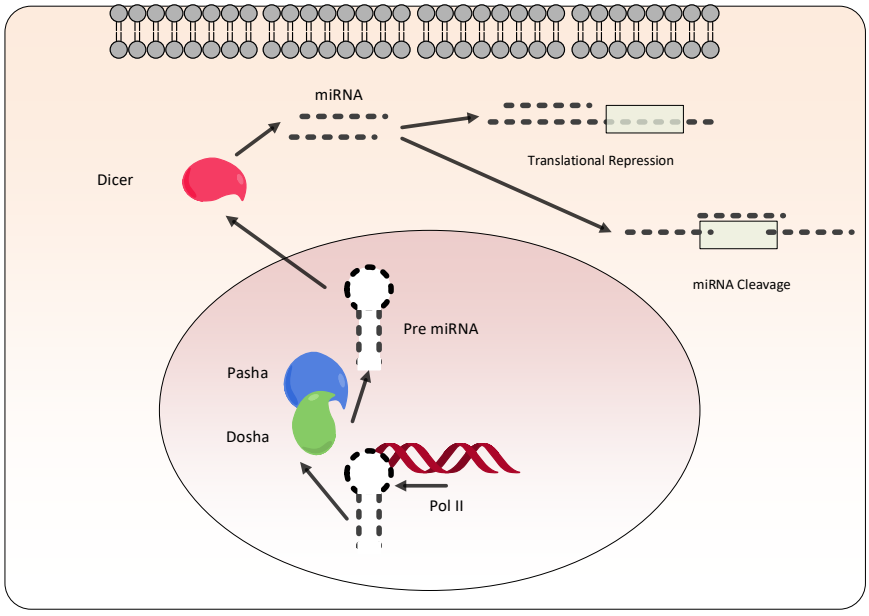
Complementary sites for miRNAs using this mechanism are generally found in the 3' untranslated regions (3' UTRs) of the target mRNA genes. miRNAs that bind to their mRNA targets with perfect (or nearly perfect) complementarity induce target-mRNA cleavage (lower right). miRNAs using this mechanism bind to miRNA complementary sites that are generally found in the coding sequence or open reading frame (ORF) of the mRNA target.



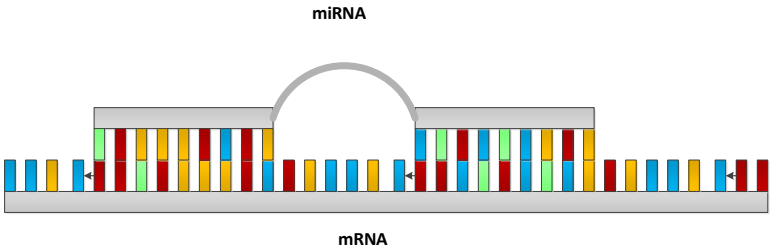
We summarize this process as follows. This is a canonical example which adheres to the original understanding.



Now from the paper we have the more detailed description where we show how miRNA can interfere with RNA translation by either inhibiting it or by slicing the RNA and in turn also inhibiting it. We depict that below. Note the two final operations on the miRNA, cleavage and repression.



An example of the miRNA binding is shown below:



This provides a rudimentary presentation of miRNA generation.

2.2 VARIANCES

Yang and Li had presented a discussion of variants on the miRNA description above. In the canonical form above, the generation is Drosha dependent and Dicer dependent. There are other modes of generation as well. One is Drosha independent – Dicer dependent and the other is Drosha independent – Dicer independent.

We summarize the Yang and Li results in the following Table:

	Dicer Dependent	***Dicer Independent***
Drosha Dependent	*Canonical animal miRNAs are generated through cleavages of hairpin precursors by RNase III enzymes. In the nucleus, the single strand-double strand junction of the pri-miRNA hairpin is recognized the catalytic site of the RNase III enzyme Drosha. This cleavage exported to the cytoplasm, where it is cleaved toward the terminal loop end by the RNase III enzyme Dicer. The duplexes are loaded into miRNA-class Argonaute effectors. One of the duplex strands is preferentially retained in Ago to form the functional RNA-induced silencing complex.*	*Pri-mir is processed by Drosha/DGCR8, and the resulting pre-mir-451 is directly incorporated to Ago2. The Slicer activity of Ago2 cleaves the arm of pre-mir, giving rise to ac-pre-mir, which is further resected by an as yet unknown mechanism to generate mature miR.*
Drosha Independent	*Mirtrons are short intronic hairpins that are excised by splicing and linearized by lariat debranching; tailed mirtrons require further resection by nucleases. RNA pol III-transcribed MHV68 tRNA-shRNA fusions are processed into pre-miRNA-like hairpins Endo-shRNAs without lower stems for Drosha/DGCR8 processing may derive from pol III transcription or cleavage by as yet unknown endo- or exonucleases. These noncanonical miRNAs, are incorporated to Ago1–4. Endogenous substrates with extended dsRNA character, including hpRNAs, transposable elements, antisense pseudogenes and natural antisense transcripts are directly cleaved by Dicer to generate siRNAs. These may potentially sort to all of the mammalian Agos, but presumably only those that load Ago2 can fulfill target slicing.*	

There also is extensive considerations regarding the miRNA-mRNA interactions. Cloonan has recently done work on this area. Cloonan has especially raised the issues we presented above regarding just how the miRNA interact with the mRNA. Cloonan notes:

One thing that is becoming clear with the uncovering of these different interaction types is that miRNAs stably interact with hundreds to thousands of mRNAs, and some consideration needs to be given to separating biological noise from functionally-driven signal. Differentiating direct from indirect consequences of miRNA action makes it easier to specifically examine the mechanisms by which miRNAs interact with their mRNA targets, but this requires more scientific rigor if it is to disentangle the functional consequences of those interactions. Issues of miRNA target detection and miRNA mode of action are intertwined with discussion of the major effect and roles of miRNAs in the cell. And, as our understanding of miRNA biology improves, so too will our ability to predict and model target interactions.

Although prediction tools have strongly dictated the way we studied miRNAs in the past, their relevancy will change as our repertoire of experimental tools improve. This will in turn shape our understanding of how, where, and why miRNAs function, ideally converging on a model that incorporates all of the data, not just those that are convenient.

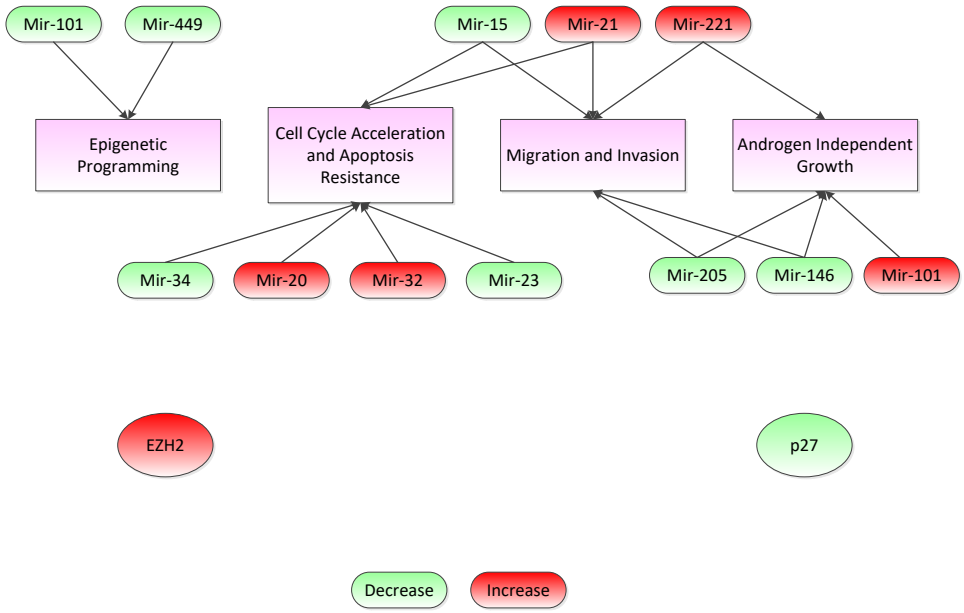
Namely, understanding the issues Cloonan presents one can understand that the simple model of miRNA and mRNA interference is substantially more complex than at first glance.

2.3 CANCER IMPLICATIONS

There has been a great amount of research regarding the impact of miRNA on cancer and especially on PCa. miRNAs may downregulate tumor suppressor genes such as PTEN. This has been seen in miRNA 21. Colin and Croce have provided several review article regarding miRNA and their influence on cancers. They argue that miRNA alterations are heavily involved in the initiation of many cancers. Their focus had been on CLL, chronic lymphocytic leukemia, and its initiating miRNAs, miR 15 and miR 16. Coppola et al (2010) provide a detailed summary of miRNAs and PCa.

2.3.1 Functions

The graphic from Coppola et al is shown below where it depicts a collection of miRNAs which impact various parts of the PCa process.



For example miR34 can cause the activation and recapitulate p53 which in turn induces cell cycle arrest and apoptosis. Loss of the miR-34 can result in the impairment of the p53 control of apoptosis and permit the cells to proliferate. Coppola et al perform a detailed analysis of all of the above related miRNAs and their resultant impact on PCa. miR-21 up-regulation leads to PTEN loss and thus is an oncogene.

Recent work by Poliseno et al has shown that PTEN can be down regulated via miR-106b. It had already been known that PTEN could be down-regulated by miR-22, miR-25 and miR-302. Their work demonstrated that miR-22 and miR-106b are overexpressed in PCa miR-106b is an intronic miRNA. The work of Poliseno thus has demonstrated a proto-oncogenic miRNA dependent network that regulates PTEN and thus can have a significant role in initiating PCa.

Micro RNAs are regulators of mRNA, the post transcriptional result which is then used to generate via translation the operative protein. Currently there are nearly 1,000 identified miRNAs. They are generally 22 nucleotides long, short segments, and they usually target specific mRNA and silence it. Each one of the miRNA may act upon many mRNAs.

As He and Hannon state:

Non-coding RNAs participate in a surprisingly diverse collection of regulatory events, ranging from copynumber control in bacteria[1] to X-chromosome inactivation in mammals[2].MicroRNAs (miRNAs) are a family of 21–25-nucleotide small RNAs that, at least for those few that have characterized targets, negatively regulate gene expression at the post-transcriptional leve.

Members of the miRNA family were initially discovered as small temporal RNAs (stRNAs) that regulate developmental transitions in Caenorhabditis elegans[6]. Over the past few years, it has become clear that stRNAs were the prototypes of a large family of small RNAs, miRNAs, that now claim hundreds of members in worms, flies, plants and mammals.

The functions of miRNAs are not limited to the regulation of developmentally timed events. Instead, they have diverse expression patterns and probably regulate many aspects of development and physiology. Although the mechanisms through which miRNAs regulate their target genes are largely unknown, the finding that at least some miRNAs feed into the RNA INTERFERENCE (RNAi) pathway has provided a starting point in our journey to understand the biological roles of miRNAs.

miRNAs are simple yet complex entities and key players in the epigenetics which control gene expression.

It is clear from the above that miRNAs can positively and negatively impact many elements in the pathways we have considered in HGPIN and PCa. Coppola et al review several of the key ones. For example:

- miR-146: Down regulates the AR.
- miR-34: Can recapitulate p53 resulting in apoptosis and arrest.
- miR-23: can result in c-myc overexpression and cell proliferation.

In a recent paper by Poliseno et al they have identified several others:

- miR-106b: Down-regulates PTEN and triggers PIN in murine models.
- miR-22, miR-25, miR-302: Down-regulating of PTEN.

Similarly the papers by Petrocca et al and that by Calin and Croce detail many of the miRNAs and their impacts on many cancers. As seen in the above graphic these are but a few in the overall targeting of PCa control genes. As Coppola et al state:

The hypothesis that miRs can be regarded as new broad-spectrum oncogenes or tumor suppressor genes has opened a revolutionary field of research with exciting diagnostic and therapeutic perspectives.

The compelling hint of a widespread miR deregulation in cancer pathogenesis came from the analysis of the genomic distribution of 186 miR. In this study, it was demonstrated that more than half of them mapped in cancer-associated genomic regions, namely in chromosomal sites prone to deletions, amplifications or recombinations. These aberrations can result in miR down- or up-regulation, conferring selective advantages to mutated cells.

Additional mechanisms of miR deregulation include altered expression of miRs as a consequence of excessive or deficient processing; aberrant transcription of the precursors by epigenetic silencing of miR promoters or as a result of the activity of oncogenic transcription factors; and more rarely, point mutations in mature miRs or in target sequences that can interfere with normal target recruitment

The problem that we will have in any modeling of HGPIN and PCa is not only do we have issues regarding the somewhat well-known genes but the impact of the epigenetic factors is unknown, complex, and possibly random.

Furthermore miRNAs can act in a positive or negative manner depending upon the cell and the activated networks in the cell. From Croce (2009) we have:

Importantly, miRNAs should not be described as oncogenes or tumor suppressor genes, unless the tissue or cell type involved in their action is specified. For example, miR-221 and miR-222 target an oncogene, KIT, and inhibit the growth of erythroblastic leukaemia[30], and therefore function as tumor suppressors in erythroblastic cells. but they also target at least four important tumor suppressors −phosphatase and tensin homologue (PTEN), p27, p57 and tissue inhibitor of metalloproteinases 3 (TIMP3) −and function as oncogenic miRNAs by suppressing these tumor suppressors in various human solid tumours[31] (TABLE 1). Therefore, before describing an miRNA as a tumor suppressor or an oncogene, it is necessary to specify in which cell or tissue, as cellular context is crucial for the function of miRNAs….

Recent work on miR-34 has demonstrated its impact on p53 (Rakhlin et al) and the fact that miR-34 significantly mediates the role of p53 in apoptosis in AR dependent PCa.

As Sevli et al state:

The miRNAs have critical functions in gene expression and their dysregulation may cause tumor formation and progression. Today, it is known that tumors possess widespread deregulated miRNA levels. Over-expression or down-regulation of specific miRNAs in different tumor types make them potential therapeutic targets and diagnostic markers. Up-regulated miRNAs

inhibiting tumor suppressor genes in tumor cells are commonly termed as oncogenic miRNAs or oncomirs. The miRNAs whose down-regulation promotes tumor progression are tumor suppressor miRNAs. One type of mRNA may possibly be targeted by multiple different miRNAs with variable efficiencies. Conversely, a single miRNA may target more than one mRNA. Thus, to be able to observe a tumorigenic phenotype, some significant changes should occur in microRNome content of the cells.

2.3.2 MiRNA and Stem Cells

As we have indicated elsewhere, the concept of the cancer stem cell has received significant attention. There has also been a great deal of work on the area of linking miRNAs and the stem cell model for PCa. In a recent work by Liu et al (2011) the authors demonstrate the nexus between miR-34a and its ability to inhibit PCa stem cells by directly repressing CD44. They observe that cancer stem cells have been observed in many solid cancers by using the fact that CD44 adheres to the cell surface. PCa stem cells with enhance clonogenic and tumor initiating and metastatic capacities are often enriched with CD44+ cell population. The work of Liu et al demonstrated that the administration of miR-34a to PCa cells inhibited PCa metastasis and inhibited PCa regeneration. This is one of the first uses of miRNA as a tumor suppressor.

In a recent paper by Xia (2008) the author states:

The key characteristics of stem cells are that they are capable of self-renewal and differentiation. The mechanisms by which stem cells maintain self-renewal and differentiation are complicated. In the past years, protein-coding genes had been broadly investigated in stem cell self-renewal and differentiation.

Recent studies indicate miRNAs as one of the most abundant classes of post-transcriptional regulators proved to be crucial in a wide range of biological processes, which suggest that miRNAs may also play essential roles in stem cell self-renewal and differentiation. Disruption of Dicer function in murine ESs influences miRNA processing and greatly impairs their ability to differentiate …

Cancer stem cells (CSCs) are the cells within a tumor that possess the capacity to self-renew and to produce the heterogeneous lineages of cancer cells that comprise the tumor. CSCs can thus only be defined experimentally by their ability of self-renewal and tumor propagation.

The implementation of this approach explains the use of alternative terms in the literature, such as "tumor-initiating cells" to describe putative CSCs. …

The identification of growth and differentiation pathways responsible for CSC proliferation and survival will help in the discovery of novel therapeutic targets. Previous studies have shown that many signal pathways may participate in regulating CSC functions, including Wnt/β-catenin, Notch, and Sonic hedgehog homolog (SHH). The canonical Wnt cascade has emerged as a critical regulator of stem cells and activation of Wnt signalling has also been associated with various cancers …

CSC maintenance is dependent on β catenin signaling. Moreover, because Wnt/β-catenin signalling is not essential for normal epidermal homeostasis, such a mechanistic difference may thus be targeted to eliminate CSCs and consequently eradicate squamous cell carcinomas. It is therefore hypothesized that inhibition of Wnt signaling may provide an effective way to reduce the unwanted stem cell renewal which results in cancers.

Inhibition of Wnt signalling may prove to be an effective road to inhibit the uncontrolled cell renewal that drives cancer. Acting as novel and pivotal regulators of protein-encoding genes, miRNAs will have great potential in regulating CSCs' biological functions by targeting CSCs-related signal pathway molecules.

We have performed various analyses of CSCs especially for PCa. This is a critical area for ongoing research and most likely will prove quite useful.

2.3.3 Multiple miRNAs

There are a large number of miRNAs and many of them work in concert. Thus there can be a great degree of correlation between them and as such any use of classifiers may have some problematic issues. As Hayes et al have noted:

MicroRNA dysregulation in cancer was first reported in 2002, when a cluster of two microRNAs, miR-15 and miR- 16, was identified at 13q14.3, a frequently deleted region in chronic lymphocytic leukemia (CLL). This microRNA deletion was shown to act at least in part through allowing higher expression of the miR-15/16 anti-apoptotic target Bcell lymphoma 2 (BCL2). Since then it has been documented that microRNAs have roles in all of the cancer hallmarks defined by Hanahan and Weinberg, and are implicated in the clinical management of cancers at every stage.

Although many specific examples have been reported, microRNA functions fall into two broad major functional categories:

(i) homeostatic regulation of gene expression, through 'fine-tuning' of translation according to cellular requirements; and

(ii) robustness in cellular responses, which is important in cell fate decisions in which groups of microRNAs can dictate the cellular differentiation state, acting as 'locks' to maintain cell identity, often via complex reciprocal negative-feedback loops.

This is seen in some cancers, in which microRNAs associated with terminal differentiation are weakly expressed in order to promote a proliferative state. Robustness is also important in responses to stress, in which microRNAs may function as 'switches' to allow cells to adapt to transient changes in their microenvironment.

For example, in glioblastoma, low levels of glucose lead to a reduction in levels of miR-451, which is necessary for AMP-activated protein kinase (AMPK) pathway activation and cell survival. Conversely, when energy levels are sufficient, higher levels of miR-451 act to suppress

AMPK signaling, and to promote mammalian target of rapamycin (mTOR) activation and cell proliferation.

Importantly, each tumor type has a distinct microRNA signature that distinguishes it from normal tissues and other cancer types. Most cancers can be further subclassified into prognostic groups based on these signatures. MicroRNA expression, like the expression of other cancer associated genes, can be altered by chromosomal amplification/deletion, promoter methylation, and transcription factor activation.

The importance of microRNA alterations in cancer is further highlighted by the observation that many cancer cells have genetic alterations that are microRNA mechanism-specific: that is, altered target binding, processing, and post-transcriptional editing. Binding site variation in the 30UTR of the target mRNA is a common feature of cancer cells. Single nucleotide polymorphisms (SNPs) and mutations have been identified, as well as deletions of 30UTRs during mRNA splicing in cancer cells, rendering mRNAs insensitive to microRNA regulation.

Hayes et al listed targets for 2014 time period. These targets are target genes which the driving miRNA has influence over. We present some of these below.

MicroRNA	***Mechanism***	***Targets***
miR-1792	Oncogene/tumor suppressor gene	E2F1, HBP1, CDKN1A, NCOA3, ERa, PTEN, MECP2, HOXA5, VPS4B, MYCN, RAB14, DPYSL2, TGFBR2, TSG101, ARHGAP12, BACE1,
miR-21	Oncogene	PDCD4, PTEN, RECK, PPARa, TIMP3, FasL, TGFBR2, SERINB5, CDK2AP1, TPM1
miR-221/222	Oncogene	CDKN1B, KIT, PPP2R2A, p27kip1, CDKN1C, ERa, KIT, DDIT4, BNIP3L, ZEB2, TBK1, CREBZF, MYBL1, DKK2
let-7	Tumor suppressor gene	NIRF, NF2, CASP3, TRIM71
miR-15/16	Tumor suppressor gene	BACE1, DMTF1, C22orf5, BCL2, ARL2, CCNT2, TPPP3, VEGFA, RARS, FGF2, ZNF622, DNAJB4, PURA, SHOC2, LUZP1, FNDC3B, ITGA2, ATG9A, CA12, TMEM43, YIF1B, TMEM189, VTI1B, RTN4, TOMM34, NAA15, PNP, SRPR, IPO4, NAPg, PFAH1B2, SLC12A2, SEC24A, NOTCH2, PPP2R5C, KCNN4, UBE4A, KPNA3, RAB30, ACP2, SRPRB, EIF4E, ABCF2, TPM3, ARHGDIA, GALNT7, LYPLA2, CHORDC1, TMEM109, LAMC1, EGFR, GPAM, ADSS, PPIF, RFT1, TNFSF9, IGF2R, TXN2, GFPT1, SLC7A1, SQSTM1, PANX1, UTP15, NPR3, SLC16A3, PTGS2, HARS, LAMTOR3, HSPA1B
miR-200	Tumor suppressor gene	ZEB1, CTNNB1, BAP1, GEMIN2, PTPRD, WDR37, KLF11, SEPT9, HOXB5, ERBB2IP. KLHL20, FOG2, RIN2, RASSF2, ELMO2, TCF7L1, VAC14, SHC1, SEPT7, FOG2
miR-34	Tumor suppressor gene	SIRT1, BCL2, YY1, MYC, CDK6, CCND1, FOXP1, HNF4a, CDKN2C, ACSL4, LEF1, ACSL1, MTA2, AXL, LDHA, HDAC1, CD44, BCL2, E2F3

We examine some more recent data latter. The difficulty with the above is twofold. First it appears for any and all cancers. Second, the apparent downstream impacts are extensive. It raises the issue of just how the downstream impacts are activated.

As Croce noted:

MicroRNA genes, unlike other genes involved in cancer, do not encode proteins. Instead, the products of these genes consist of a single RNA strand of about 21 to 23 nucleotides; their function is to regulate gene expression.

A microRNA molecule can anneal to a messenger RNA (mRNA) containing a nucleotide sequence that complements the sequence of the microRNA. In this way, the microRNA blocks protein translation or causes degradation of the mRNA. Examples of the role microRNA plays in cancer pathophysiology involve miR-15a and miR-16-1, which are deleted or down-regulated in most indolent cases of chronic lymphocytic leukemia, suggesting an early event in the pathogenesis of this disease.

Mapping of numerous microRNA genes has shown that many occur in chromosomal regions that undergo rearrangements, deletions, and amplifications in cancer cells. The regions of the genome that are consistently involved in chromosomal rearrangements in cancer cells but that lack oncogenes or tumor-suppressor genes appear to harbor microRNA genes. Expression profiling of microRNA genes has revealed signatures associated with tumor classification, diagnosis, staging, and progression, as well as prognosis and response to treatment.

For example, microRNA expression profiling can distinguish between indolent and aggressive forms of chronic lymphocytic leukemia, and expression of a small panel of microRNA genes correlates with prognosis in stage 1 lung cancer. Some microRNA genes that are deregulated in chronic lymphocytic leukemia have germ-line or somatic mutations in a microRNA precursor that affect the processing of short single-stranded microRNA molecules.62 MicroRNA genes can be up-regulated or down-regulated in cancer cells.

The up-regulated genes function as oncogenes by down-regulating tumor-suppressor genes, whereas the down-regulated genes function as tumor suppressor genes by down-regulating oncogenes. The function of microRNA genes depends on their targets in a specific tissue. A microRNA gene can be a tumor suppressor if in a given cell type its critical target is an oncogene, and it can be an oncogene if in a different cell type its target is a tumor-suppressor gene. Up-regulation of microRNA genes can be due to amplification, deregulation of a transcription factor, or demethylation of CpG islands in the promoter regions of the gene.

For example, the ALL1 (MLL) fusion proteins of acute lymphoblastic leukemia or acute myeloblastic leukemia carrying chromosome 11q23 translocations target the Drosha nuclease complex to specific microRNA genes, including miR191, thereby enhancing the processing of their microRNA precursors. The miR191 gene is also up-regulated in numerous types of solid cancers, suggesting that it is the downstream target of signal-translocation pathways involved in cancer.

MicroRNA genes functioning as tumor suppressors can be down-regulated because of deletions, epigenetic silencing, or loss of the expression of one or more transcription factors.

Esquela-Kerscher and Slack further detail the functioning of miRNAs as drivers and inhibitors as follows:

microRNAs can function as tumour suppressors and oncogenes.

a. In normal tissues, proper microRNA (miRNA) transcription, processing and binding to complementary sequences on the target mRNA results in the repression of target-gene expression through a block in protein translation or altered mRNA stability. The overall result is normal rates of cellular growth, proliferation, differentiation and cell death.

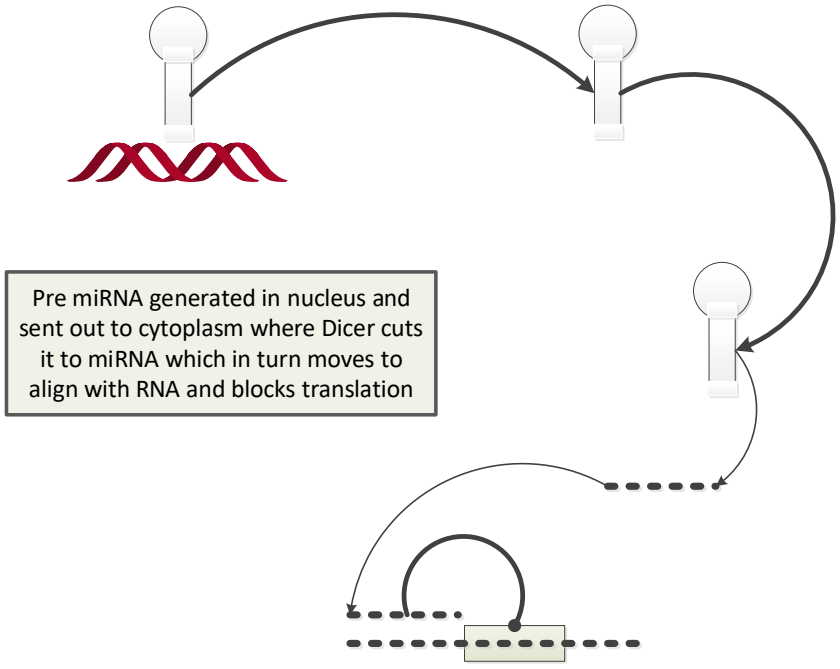
b. The reduction or deletion of a miRNA that functions as a tumour suppressor leads to tumour formation.

A reduction in or elimination of mature miRNA levels can occur because of defects at any stage of miRNA biogenesis (indicated by question marks) and ultimately leads to the inappropriate expression of the miRNA-target oncoprotein (purple squares). The overall outcome might involve increased proliferation, invasiveness or angiogenesis, decreased levels of apoptosis, or undifferentiated or de-differentiated tissue, ultimately leading to tumour formation.

The amplification or overexpression of a miRNA that has an oncogenic role would also result in tumour formation. In this situation, increased amounts of a miRNA, which might be produced at inappropriate times or in the wrong tissues, would eliminate the expression of a miRNA-target tumour-suppressor gene (pink) and lead to cancer progression.

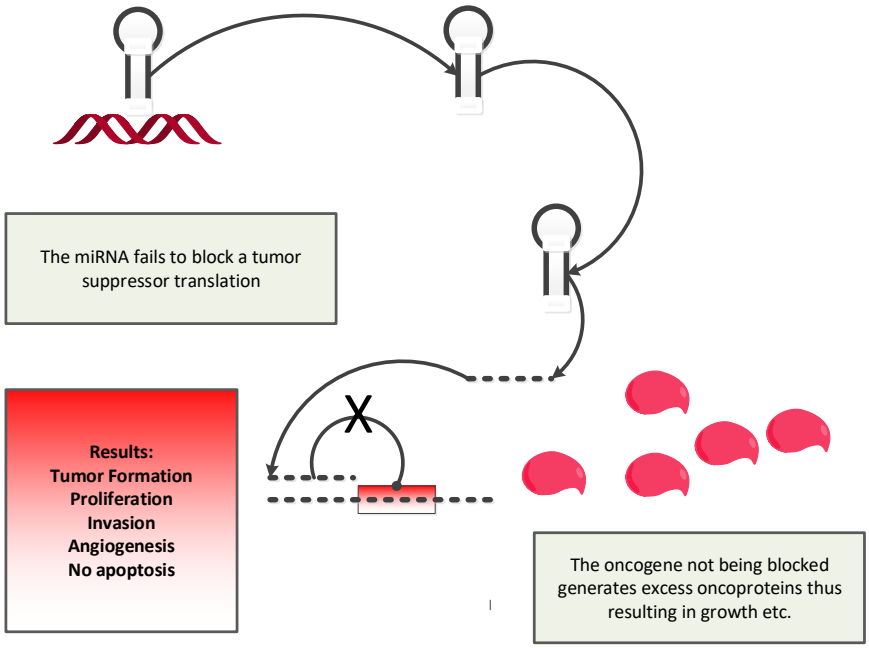
Increased levels of mature miRNA might occur because of amplification of the miRNA gene, a constitutively active promoter, increased efficiency in miRNA processing or increased stability of the miRNA (indicated by question marks). ORF, open reading frame.

We depict these three cases as follows. First, miRNA acting in a normal manner. This is below:

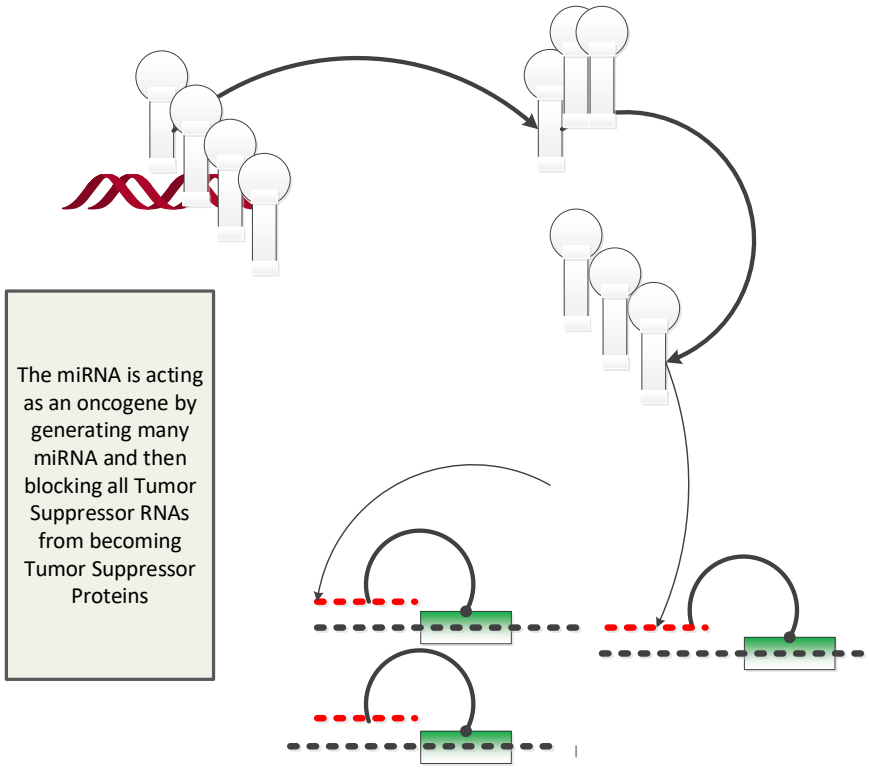


Notice above the miRNA is assumed to be a normal part of the control mechanism of the control of the conversion of the mRNA into a protein. It blocks the conversion.

Now consider the second case. Here we have an oncogene which is not blocked by the miRNA and it results in many oncoproteins as shown below.

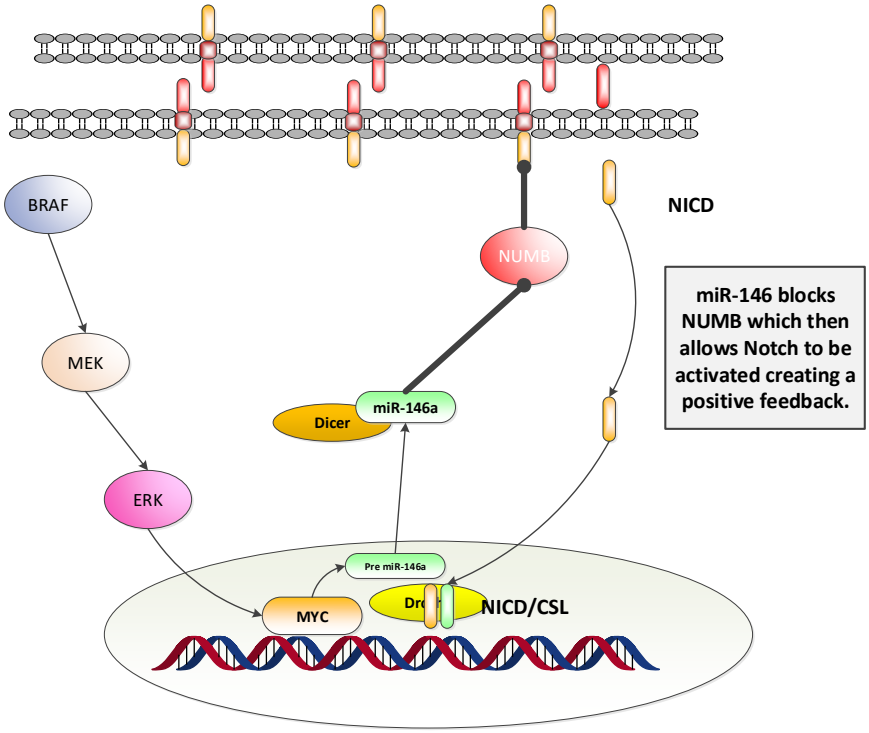


Finally in case 2 we have a massive explosion of miRNAs acting as onco activators as shown below.

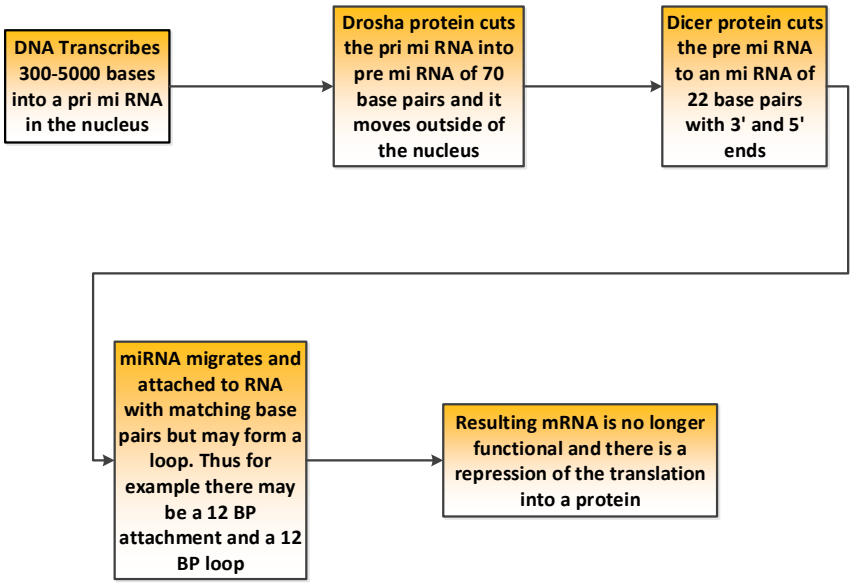


These methods demonstrate in a somewhat simple manner how the miRNA functions in the case of certain cancers. It also demonstrates how the miRNA can become a target for therapeutics.

miRNA and its ability to block mRNA functions can thus directly block a multiplicity of cancer related gene products and thus activate malignant pathways. The following figure depicts some specific gene interference.



Finally the following diagram depicts these processes. From the generation to the actual inhibition.



2.4 METASTATIC CONTROL

Finally, miRNAs have been identified as major participants in metastatic processes. As Sole and Lawrie have noted:

The first reports associating miRNAs with metastasis came in 2007, with the demonstration that miR-10b was induced by Twist1 binding and could promote metastasis in breast cancer in vitro and in vivo through targeting of Homeobox D10 (HOXD10). In the same year, also in breast cancer, let-7 was identified as a suppressor of metastasis acting to target the GTPase H-RAS and High Mobility Group AT-Hook 2 (HMGA2) gene in tumor-initiating cells, resulting in reduced proliferation and mammosphere formation in vitro and decreased metastasis in a NOD/SCID murine model.

In subsequent studies, breast cancer has remained the main focus of research investigating miRNAs in metastasis, and many studies have shown that miRNAs can act as both promoters or inhibitors of metastasis in cancer and modulate many steps of the metastatic pathway, including migration, invasion, adhesion, the epithelial–mesenchymal transition (EMT), niche conditioning and proliferation in secondary site.

2.4.1 Promotion

We can now provide some specific examples. From the perspective of promotion we have from Sole and Lawrie who categorize these as:

In common with miR-10b, many of the metastasis-promoting miRNAs that have been characterized have been identified in breast cancer. For example, miR-105 has been identified as being up-regulated in tumor cells and exosomes derived from breast cancer cells were demonstrated to breakdown vascular endothelial barriers and induce vascular permeability, thereby promoting metastasis by targeting of ZO-1, a component of cell–cell adhesion complexes in endothelial and epithelial cells. Furthermore, these authors used exosomes to reduce tight junction formation in endothelial monolayers and induce vascular permeability and metastasis in vivo. MiR-181b-3p was demonstrated to promote EMT in vitro, with its inhibition reducing the expression of mesenchymal markers, migration and invasion in highly metastatic cell lines

2.4.2 Suppressing

Secondly Sole and Lawrie discuss the suppressing function of miRNAs:

In addition to miRNAs that promote metastasis, other miRNAs negatively regulate this process and are consequently found to be down-regulated in cancer tissues and/or cell lines. For example, members of the miR-200 family (i.e., miR-200a, miR-200b, miR-200c, miR-141 and miR-429) as well as miR-205 have been shown to inhibit the expression of transcription repressors ZEB1 and ZEB2 to enhance E-Cadherin expression, thereby inhibiting EMT in breast cancer.

In a study that looked at miRNA expression in 59 of the NCI60 cell lines that had a E-Cadherin high and vimentin low (EMT inhibitory) phenotype, they observed a strong negative correlation with miR-200 expression, suggesting that this miRNA is a universal regulator of metastasis in many cancer types including lung, kidney, colon and ovarian cancer.

Specifically, miR-200b was shown to be down-regulated in triple negative breast cancer (TNBC) as a result of the recruitment of DNMT3A by MYC, which in turn binds to the miR-200b

promoter region, resulting in promoter methylation and silencing, thereby inhibiting migration, invasion and mammosphere formation in TNBC cells . Part of the miR-200 family, miR-141 is also down-regulated in prostate cancer (PC), and its ectopic expression was shown to inhibit invasion and metastasis and to convey a strong epithelial phenotype with a partial mesenchymal phenotype

2.4.3 Tumor Micro Environment

The tumor micro environment is something we have examined in detail. Whether it is the extracellular matrix or the tumor associate macrophages, these extracellular complexes are now known to play significant roles in the support of tumors and their proliferation. Again, as Sole and Lawrie then focus on the TAM. Namely they note:

MiRNAs contained in exosomes have also been shown to influence non-tumor cells in the tumor microenvironment such as tumor-associated macrophages (TAMs) that promote invasion and metastasis in cancer. For example, pancreatic cancer cells under hypoxic conditions were shown to release exosomes that contained miR-301a-3p, which was demonstrated to induce TAM polarization resulting in increased pancreatic cell migration and EMT in vitro and lung metastasis in vivo. This polarization was induced by activation of the PTEN/PI3Kγ signaling pathway.

In contrast, TAMs themselves have also been shown to secrete exosomes containing functional miRNAs that can promote metastasis. For example, exosomal miR-223 derived from TAMs of breast cancer patients were demonstrated to promote tumor cell invasion through targeting of the Mef2c-β-catenin pathway. In colon cancer, activated TAMs were shown to release exosomes containing miR-21-5p and miR-155-5p, which were demonstrated to regulate migration and invasion of colorectal cancer cells through targeting of BRG1. In addition to TAMs, cancer-associated fibroblasts (CAFs), which initiate remodeling of the extracellular matrix, thereby facilitating metastasis, can also release and respond to miRNA-containing exosomes.

This is the case for example in prostate cancer, where EV-associated miR-409 was demonstrated to promote EMT both in vitro and in vivo through down-regulation of RSU1 and STAG2. In breast cancer, tumor cells were demonstrated to secrete exosomes containing miR-122 that could induce glucose reallocation in pre-metastatic sites in fibroblast and astrocyte populations, thereby making sites more conducive to metastasizing cancer cells

2.4.4 Exosomes

We have separately examined the potential for miRNAs to be themselves a means for metastatic transfer via exosomes[1]. Specifically we have postulated a reasonable mechanism for this transmission[2].

[1] https://www.researchgate.net/publication/334429457_miRNAs_Genes_and_Cancer_Cytology

[2] https://www.researchgate.net/publication/331318495_Exosomes_and_Cancer

Jiang et al have also recently noted the relationship between exosomes and miRNAs. They state:

Through intercellular transfer of exosomal miRNA, tumor parenchymal cells can confer drug resistance to each other and enhance the invasiveness of recipient cells. Tumor cells can promote angiogenesis of endothelial cells, and endothelial cells can promote tumor cell proliferation. Immune cells are able to regulate tumor metastasis under different conditions, and tumor cells may induce immune cell dysfunction and pro-inflammatory cytokine release.

Tumor cells are capable of inducing CAF phenotype transformation of mesenchymal stem cells, and mesenchymal stem cells can be transferred to the tumor site to promote tumor metastasis or dormancy, but they also inhibit tumor growth in some cases. Adipocytes play an important role in promoting tumor cell invasion, while the effect of tumor-secreted exosomes on adipocytes has not been reported to date. Exosomal miRNA can convert normal fibroblasts into CAFs for tumor survival, and CAFs can promote tumor progression

2.4.5 Examples

Sole and Lawrie summarize their results in the following Table. As we shall note as we examine several significant malignancies these may vary from study to study. The important factor is to understand the targets. As we noted previously, targets are suppressed for the most part and thus either blocking the miRNA or up expressing the target may be a reasonable therapeutic.

miRNA	Cancer	Express.	Sample	Target
Let-7	Breast	Low	Cells & Tissue	*RAS & HMGA2*
miR-7	Breast	Low	Cells & Tissue	*FAK*
miR-10b	Breast	High	Cells & Tissue	*HOXD10*
miR-26a/b	HNSCC	Low	Cells & Tissue	*LOXL2*
miR-29 family	HNSCC	Low	Cells	*LAMC2 & ITGA6*
miR-29 family	HNSCC	Low	Cells & Tissue	*LOXL2*
miR-29 family	ccRCC	Low	Cells & Tissue	*LOXL2*
miR-29a	Breast	High	Cells & Tissue	*SUV420H2*
miR-29c	Lung	Low	Cells	*Integrin* β1 *& MMP2*
miR-31	Breast	Low	Cells	*Integrin* α *subunits*
miR-31	Breast	Low	Cells & Tissue	*WAVE3*
miR-33b	Breast	Low	Cells & Tissue	*HMGA2, SALL4 & Twist1*
miR-34	CRC	Low	Cells	*ZEB1 & Slug*
miR-34a/b/c	CRC	Low	Cells	*Snail*
miR-34a	Breast	Low	Cells & Tissue	*CXCL10*
miR-34c	Breast	Low	Cells & Tissue	*GIT1*
miR-93	Endometrial	High	Cells & Tissue	*FOXA1*
miR-96	Breast	High	Cells & Tissue	*PTPN9*
miR-96	HCC	High	Cells & Tissue	*ephrinA5*
miR-101	Ovarian	Low	Cells	*ZEB1 & ZEB2*
miR-101	NSCLC	Low	Cells & Tissue	*ZEB1*
miR-105	Breast	High	Cells, Tissue & Serum	*ZO-1*
miR-124	Breast	Low	Cells & Tissue	*ZEB2*
miR-124	Breast	Low	Cells & Tissue	*IL-11*
miR-124	CRC	Low	Cells & Tissue	*ROCK1*
miR-128-3p	ESCC	Low	Cells & Tissue	*ZEB1*
miR-130a	Breast	Low	Cells	*FOSL1*
miR-132	NSCLC	Low	Cells & Tissue	*ZEB2*

miRNA	Cancer	Express.	Sample	Target
miR-132	CRC	Low	Cells & Tissue	*ZEB2*
miR-135a	Breast	High	Cells & Tissue	*HOXA10*
miR-135a	HCC	High	Cells & Tissue	*FOXO1*
miR-135a	Gastric	Low	Cells & Tissue	*ROCK1*
miR-135b	Breast	High	Cells & Tissue	*APC*
miR-138	Breast	Low	Cells & Tissue	*ROCK1*
miR-139-5p	Glioblastoma	Low	Cells & Tissue	*ZEB1 & ZEB2*
miR-141	Breast	Low	Cells & Tissue	*HIPK1*
miR-141	Prostate	Low	Cells & Tissue	*Rho GTases, CD44 & EZH2*
miR-150	Breast	Low	Cells & Tissue	*HMGA2*
miR-154	Breast	Low	Cells & Tissue	*E2F5*
miR-181b	Breast	High	Cells & Tissue	*YWHAG*
miR-182	HCC	High	Cells & Tissue	*ephrinA5*
miR-186-5p	CRC	Low	Cells	*ZEB1*
miR-190	Breast	Low	Cells	*STC2*
miR-190	Breast	Low	Cells & Tissue	*SMAD2*
miR-194	Ovarian	High	Cells & Tissue	*PTPN12*
miR-197	HCC	High	Cells & Tissue	*Axin-2, NKD1 & DKK2*
miR-200	Breast	Low	Cells & Tissue	*ZEB1 & ZEB2*
miR-200a/b	Breast	Low	Cells	*ZEB1 & ZEB2*
miR-200c	Breast	Low	Cells	*CRKL*
miR-200c	Breast	Low	Cells	*ZEB1*
miR-200c	Breast	Low	Cells & Tissue	*HIPK1*
miR-203	HNSCC	Low	Cells & Tissue	*LASP1, SPARC & NUAK*
miR-203	Breast	Low	Cells	*Slug*
miR-203	CRC	Low	Cells & Tissue	*EIF5A2*
miR-203	Melanoma	Low	Cells & Tissue	*Slug*
miR-203	Ovarian	Low	Cells & Tissue	*BIRC5*
miR-203	Gastric	Low	Cells & Tissue	*Annexin A4*
miR-205	Breast	Low	Cells	*ZEB1*
miR-205	Breast	Low	Cells & Tissue	*ZEB1 & ZEB2*
miR-210	Breast	High	Cells & Tissue	*E-Cadherin & Snail*
miR-215	NSCLC	Low	Cells & Tissue	*ZEB2*
miR-218	HNSCC	Low	Cells & Tissue	*LOXL2*
miR-374a	Breast	High	Cells & Tissue	*WIF1, PTEN & WNT5A*
miR-409-3p	Osteosarcoma	Low	Cells & Tissue	*ZEB1*
miR-429	Breast	Low	Cells	*ZEB1 & ZEB2*
miR-504	NSCLC	Low	Cells & Tissue	*LOXL2*
miR-508-3p	Breast	Low	Cells & Tissue	*ZEB1*
miR-520c	Gastic	High	Cells & Tissue	*IRF2*
miR-574-5p	NSCLC	High	Cells, Tissue & Serum	*PTPRU*
miR-577	Gastric	High	Cells & Tissue	*SDPR*
miR-641	Cervical	Low	Cells & Tissue	*ZEB1*
miR-708-3p	Breast	Low	Cells & Tissue	*ZEB1, CDH2 & Vimentin*
miR-1204	Breast	High	Cells & Tissue	*VDR*
miR-1269a	Colorectal	High	Cells & Tissue	*Smad7 & HOXD10*
miR-8084	Breast	High	Cells, Tissue & Serum	*ING2*

3 PROSTATE

We have examined prostate cancer (PCa) and miRNAs extensively for the past decade[3]. Prostate cancer is a pervasive malignancy with an increasing incidence until the USPTF indirectly and baselessly banned indirectly the PSA testing[4]. However PCa can be considered to fall into two classes. One, the largest, is a slow growing malignancy which can take years to develop. The second is a highly aggressive form leading to death is a short period. As of this writing there is no definitive marker or markers to differentiate the two. Thus using miRNAs may be an added benefit.

3.1 MIRNA AND PCA

Let us begin with an overview of miRNAs in PCa. As Luu et al have noted:

…recent studies indicated the dysregulation of miRNA profiles in PCa based on high-throughput methodologies (i.e., microarrays, RNA-Seq, proteomic arrays). Further, miRNA expression profiles may help to distinguish between normal and tumor tissues. miRNA profiles appear to have a better accuracy to differentiate between tumor and normal tissues when compared to mRNA profiling. Expression profiles of miRNAs often correspond with clinic-pathological parameters and predict patient outcome or response to treatment.

These observations underscore the potential of miRNAs as new diagnostic or prognostic biomarkers. Additionally, the oncogenic or tumor suppressive functions of miRNAs suggested that miRNAs may be new candidates for cancer drug treatment.

How effective these can be is yet uncertain. We shall focus on this later in this note.

Beyond that, miRNAs has also been shown to be more stable than the mRNA and are therefore more suitable to be measured in formalin-fixed paraffin-embedded (FFPE) samples. The stability of miRNAs also makes miRNAs better candidates for blood-based marker. Dysregulation of miRNA in cancer could be caused by several genomic aberrations.

These genomic anomalies may include, chromosomal modification, such as translocation, change of transcriptional factors and aberrant expression, epigenetic alterations, and changes in miRNA processing.

Several studies reveal miR-155 is induced at the transcriptional level by transforming growth factor β (TGFB1)/Smad, nuclear factor-κB (NFKB1) and activator protein-1 (AP1) family transcription factors (JUN/JUNB/JUND/FOS/FOSB) through direct interaction with the miR-155 promoter.

[3] https://www.researchgate.net/publication/325046881_PCa_mir34_p53_MET_and_Methylation

[4] https://www.researchgate.net/publication/264960277_Prostate_Cancer_A_Systems_Approach

Looking for non-invasive tests is at the top of the list which are needed. As de Souza et al note:

Circulating nucleic acids (cfNAs) are free DNA and RNA *molecules in the plasma, serum, and urine of cancer patients and healthy individuals, which can be used as minimally invasive diagnostic tools. These molecules are found in body fluids in free forms or bound to proteins or exosome-associated. cfNAs originate from necrotic and apoptotic cells or are secreted by several cell types. These molecules are stable and exhibit potential to be used as biomarkers; however, a limited number of studies have focused on circulating RNAs (cfRNAs) in PCa . cfNAs may be served as a "liquid biopsy," which would be useful for diagnostic applications without the need for biopsy.*

Furthermore, these molecules are powerful tools for monitoring the disease and to evaluate the efficacy of treatment in a rapid non-invasive technique.

Among cfRNAs, the circulating microRNAs (cfmiRNAs) have been intensively studied. *miRNAs are small non-coding RNA molecules of approximately 22 nucleotides long; acting as post-transcriptional regulators and exhibiting preferential binding to the 30 untranslated region (3'UTR) of mRNAs. A few studies have focused on cfmiRNAs as new attractive cancer biomarkers, with miR-141 and miR-375 being the most promising miRNAs described in prostate cancer.*

As we will note there is always a claim for circulating miRNAs. They do not break down and they have a rich history of being reflective of cellular activity. However they are not dispositive singularly.

However, the role of these aberrant circulating miRNAs is unclear. Recently, Wang et al. reported the involvement of the miR-410-5p in the modulation of the communication between cancer cells and dendritic cells. Previously, the authors described its potential as serum diagnostic marker in prostate cancer. Nevertheless, circulating mRNAs (cfmRNAs) have been poorly explored in cancer research. In prostate cancer, the genes telomerase reverse transcriptase (hTERT) and bone morphogenetic protein-6-specific (BMP6) are reported as biomarkers for PCa diagnosis.

3.2 TARGET MIRNAS

The authors then delineate the following putative miRNA targets.

MicroRNA	***Predicted target genes***
hsa-miR-133a-3p	*AMACR, BCL2, COL1A1, NKX3-1, SIM2*
hsa-miR-133b	*AMACR, BCL2, COL1A1, NKX3-1, SIM2*
hsa-miR-143-3p	*AMACR, BCL2, COL1A1, GOLM1, MPM11, NKX3-1, OR51E2, SIM2*
hsa-miR-183-5p	*AMACR, COL1A1, FOXA1, MPM11, NKX3-1, TRPM8*
hsa-miR-200b-3b	*AMACR, BCL2, GOLM1, OR51E2, SIM2, TRPM*
hsa-miR-200c-3p	*AMACR, BCL2, FOXA1, GOLM1, OR51E2, SIM2, TRPM8*
hsa-miR-205-5p	*AMACR, BCL2, COL1A1, GOLM1, MPM11, NKX3-1, SIM2, TRPM8*
hsa-miR-375	*AMACR, BCL2, COL1A1, FOXA1, GOLM1, NKX3-1, SIM2, TRPM8*

As we shall see, almost every author has their list of "the usual suspects". At present there is no dispositive list based upon adequate clinical evidence, no less a clear understanding of the genomic networking effects. In a recent paper by Cheng et al (2014) they state:

The miR-34 family was originally found to be a direct target of p53 and is a group of putative tumor suppressors. Surprisingly, mice lacking all mir-34 genes show no increase in cancer formation by 18 months of age, hence placing the physiological relevance of previous studies in doubt.

Here, we report that mice with prostate epithelium-specific inactivation of mir-34 and p53 show expansion of the prostate stem cell compartment and develop early invasive adenocarcinomas and high-grade prostatic intraepithelial neoplasia, whereas no such lesions are observed after inactivation of either the mir-34 or p53 genes alone by 15 months of age.

Consistently, combined deficiency of p53 and miR-34 leads to acceleration of MET-dependent growth, self-renewal, and motility of prostate stem/ progenitor cells.

Our study provides direct genetic evidence that mir-34 genes are bona fide tumor suppressors and identifies joint control of MET expression by p53 and miR-34 as a key component of prostate stem cell compartment regulation, aberrations in which may lead to cancer

This is a murine model which putatively demonstrates that a blocking of both miR-34 and p53 leads to PCa. Specifically, this is MET pathway dependent growth. As noted in Bioscience Technology[5]:

Previous research at Cornell and elsewhere has shown that another gene, called p53, acts to positively regulate miR-34. Mutations of p53 have been implicated in half of all cancers. Interestingly, miR-34 is also frequently silenced by mechanisms other than p53 in many cancers, including those with p53 mutations.

[5] http://www.biosciencetechnology.com/news/2014/03/gene-family-proven-suppress-prostate-cancer also http://www.news.cornell.edu/stories/2014/03/gene-family-proven-suppress-prostate-cancer

The researchers showed in mice how interplay between genes p53 and miR-34 jointly inhibits another cancer-causing gene called MET. In absence of p53 and miR-34, MET overexpresses a receptor protein and promotes unregulated cell growth and metastasis.

This is the first time this mechanism has been proven in a mouse model, said Alexander Nikitin, a professor of pathology in Cornell's Department of Biomedical Sciences and the paper's senior author. Chieh-Yang Cheng, a graduate student in Nikitin's lab, is the paper's first author.

In a 2011 Proceedings of the National Academy of Sciences paper, Nikitin and colleagues showed that p53 and miR-34 jointly regulate MET in cell culture but it remained unknown if the same mechanism works in a mouse model of cancer (a special strain of mice used to study human disease).

The findings suggest that drug therapies that target and suppress MET could be especially successful in cancers where both p53 and miR-34 are deficient.

Also, the number of stem cells in mice with both p53 and miR-34 silenced increased substantially compared with control mice or mice with only miR-34 or p53 independently silenced.

"These results indicated that together [miR-34 and p53] regulate the prostate stem cell compartments," said Nikitin.

Cancer stem cell regulation is an important factor. However there is still a great deal of uncertainty and lack of clarity regarding these artifacts.

This is significant, as cancer frequently develops when stem cells become unregulated and grow uncontrollably, he said.

Researchers further found that p53 and miR-34 affect stem cell growth by regulating MET expression. In absence of p53 and miR-34, MET is overexpressed, which leads to uncontrolled growth of prostate stem cells and high levels of cancer in these mice.

From Tang's Lab at MD Anderson we have[6] (see Liu et al):

Cancer stem cells (CSCs), or tumor-initiating cells, are involved in tumor progression and metastasis. MicroRNAs (miRNAs) regulate both normal stem cells and CSCs and dysregulation of miRNAs has been implicated in tumorigenesis6. CSCs in many tumors—including cancers of the breast, pancreas, head and neck, colon, small intestine, liver, stomach, bladder and ovary—have been identified using the adhesion molecule CD44, either individually or in combination with other marker(s).

Prostate CSCs with enhanced clonogenic17 and tumor-initiating and metastatic capacities are enriched in the CD44+ cell population, but whether miRNAs regulate CD44+ prostate cancer cells and prostate cancer metastasis remains unclear. Here we show, through expression

[6] http://staging-www.nature.com/nm/journal/v17/n2/full/nm.2284.html

analysis, that miR-34a, a p53 target was underexpressed in CD44+ prostate cancer cells purified from xenograft and primary tumors.

Enforced expression of miR-34a in bulk or purified CD44+ prostate cancer cells inhibited clonogenic expansion, tumor regeneration, and metastasis. In contrast, expression of miR-34a antagomirs in CD44− prostate cancer cells promoted tumor development and metastasis. Systemically delivered miR-34a inhibited prostate cancer metastasis and extended survival of tumor-bearing mice.

We identified and validated CD44 as a direct and functional target of miR-34a and found that CD44 knockdown phenocopied miR-34a overexpression in inhibiting prostate cancer regeneration and metastasis. Our study shows that miR-34a is a key negative regulator of CD44+ prostate cancer cells and establishes a strong rationale for developing miR-34a as a novel therapeutic agent against prostate CSCs.

Luu et al have identified a large number of miRNAs as follows by category. In the following lists we have the location of the miRNA, the type it is by the action it takes, and the list of specific miRNAs:

3.2.1 Risk and Diagnostic:

Location	***Action***	***miRNA***
Tissue	Risk/Diagnostic	Let-7b-g, 26a-b, 29a-c, 30a-e, 99a-b, 125a-b
Tissue	Risk/Diagnostic	miR-205

3.2.2 Diagnostic

Location	***Action***	***miRNA***
Tissue/Serum	Diagnostic	let7i, 16, 26a, 195
Tissue	Diagnostic	miR-143, 145, 375
Tissue	Diagnostic	miR-145
Tissue/Urine	Diagnostic	miR-205, 221, 99b
Tissue	Diagnostic	miR-182-5p
Tissue	Diagnostic	miR-361-3p, 133b, 221, 203
Tissue/Serum	Diagnostic	miR-200b, 200c, 375
Tissue	Diagnostic	Up: miR-375, 663b, 615-3p, 425-5p, 663a, 182-5p, 183-5p. Down: miR-205-5p, 221-3p, 222-3p, 376c-3p, 136-5p, 455-3p, 455-5p, −154-5p
Serum	Diagnostic	miR-141
Serum	Diagnostic	miR-16, 92a, 103, 107
Serum	Diagnostic	miR-26b, 223, 874, 1274a
Plasma	Diagnostic	miR-21, 141, 221
Serum	Diagnostic	miR-141, 298, 346, 375

Plasma	Diagnostic	let7c, let7e, 30c, 622, 1285
Serum	Diagnostic	miR-210
Serum	Diagnostic	miR-141, 375
Serum	Diagnostic	miR-141
Plasma	Diagnostic	let-7c, 375
Urine	Diagnostic	miR-182-5, 484
Urine	Diagnostic	miR-483-5p, 1275, 1290

3.2.3 Risk and Progression

Location	***Action***	***miRNA***
Tissue	Risk/Progression	let-7a-d, let-7f, 19b, 145, 184, 198, 202, 210
Tissue	Risk/Progression	miR-1, 32, 101, 106b, 182, 200a, 200b, 494, 520 h
Tissue	Progression	miR-221, 222
Tissue	Progression	miR-2, 10, 125b, 224
Tissue	Risk/Progression	miR-23b, 100, 135b, 145, 194, 221, 222
Tissue	Progression	miR-9*, 16, 221, 222, 331-3p, 145, 551a
Tissue	Progression	miR-143
Tissue	Risk/Progression	miR-96, 205
Tissue	Progression	miR-20a
Tissue	Progression	miR-128
Tissue	Progression	miR-221
Tissue	Risk/Progression	miR-34c
Tissue/Serum	Progression	miR-141, 375
Tissue	Progression	miR-151
Tissue	Progression	let7c, miR-100, miR-145, miR-191
Tissue	Progression	miR-1, 133a, 133b, 143, 143*, 145, 145*, 204, 221, 222
Tissue	Progression	miR-21
Tissue	Progression	miR-182-5p
Tissue	Progression	miR-21
Tissue	Progression	miR-19a, miR-374b
Tissue	Progression	miR-224, 452
Tissue/Serum	Progression	miR-19, miR-345, miR-519c-5p
Tissue	Progression	Let-7c
Serum	Progression	miR-21
Plasma	Progression	miR-21, miR-221
Plasma	Progression	miR-20a, miR-21, miR-145, miR-221
Plasma	Progression	miR-21, −126, −141, −151-3p, −152, −200c,-375, −423-3p
Serum	Progression	miR-141, miR-375, miR-378, miR-409-3p

3.2.4 Diagnostic and Progression

Location	***Action***	***miRNA***
Tissue	Diagnostic/Progression	miR-21
Tissue	Diagnostic/Progression	miR-96, miR-182, miR-183
Serum/Urine	Diagnostic/Progression	miR-181a-2, miR-625, miR-107, miR-574-3p, miR-20a miR-23a, miR-624
Tissue	Diagnostic/Progression	miR-224
Tissue	Diagnostic/Progression	miR-96-5p, miR-183-5p, miR-145-5p, miR-221-5p
Tissue	Diagnostic/Progression	Up: miR-122, miR-335, miR-184, miR-193, miR-34, miR-138 Down: miR-373, −9, −198, −144 -215, −96, −222, −148, −92, −27, −125, −126, −27
Tissue/Serum	Diagnostic/Progression	miR-187 and miR-182
Serum	Diagnostic/Progression	let-7a, −24, −26b, −30c, −93, −100, −103, −106a, −107, −130b, −146a, −223, −451, −874,
Tissue	Diagnostic/Progression	miR185-5p, miR-221-3p, miR-326

3.2.5 Treatment Response

Location	***Action***	***miRNA***
Tissue	Treatment Response	miR-23b, miR-220, miR-221, miR-222, and miR-205
Tissue	Treatment Response	miR-152
Plasma	Treatment Response	miR-141
Serum	Treatment Response	miR-210
Blood	Treatment Response	miR-99a

3.2.6 Additional Targets

Now Vanacore et al have also listed target miRNAs by function as follows. First the following are oncogenic.

miR-21	promotes tumor invasiveness and induces castration-resistance phenotype.
miR-221/miR-222	enhance cell proliferation, invasion, cell survival, increase clonogenicity and enhance tumorigenicity *in vivo*.
miR-141	is important in androgen-dependent and in metastatic castration-resistant.
miR-375	is important for an early diagnosis.

miR-18a	promotes cancer progression.
miR-4534	induces pro-cancerous characteristics in non-cancer cell line.
miR-650	suppresses the cellular stress response 1 (CSR1) expression.
miR-32	inhibits apoptosis and enhances proliferation.
miR-106/miR-25	facilitate tumor progression.
miR-125b	enhances cell proliferation and inhibits apoptosis.

The following are tumor suppressors:

miR-34a	induces cell-cycle arrest, cell senescence and apoptosis and inhibits cell proliferation and cell invasion.
miR-145	inhibits invasion, migration and arrests cell cycle.
miR-224	inhibits invasion and migration of PCa cells.
miR-452	regulates cell cycle, cellular adhesion and motility.
miR-200b	inhibits PCa cell growth and invasion.
miR-382	inhibits PCa cell proliferation, migration, invasion and metastasis.
miR-372	inhibits proliferation, migration and invasion of DU145 cells.
miR-17-92a	decreases cell cycle regulatory proteins and the expression of mesenchymal markers.
miR-27a	suppresses MAP2K4 in PCa cell.
has-miR-135-a-1	inhibits cell growth, cell cycle progression, migration, invasion, and xenograft tumor formation.
miR-204-5p	promotes apoptosis by targeting BCL2 in PCa cell.
miR-30a	reduces expression of cell cycle protein, cyclin E2.
let-7 miRNAs	regulate cell cycle, cell migration, cell proliferation and epithelial-to-mesenchymal transition progression.
miR-133/miR-146a	suppress tumor progression via targeting EGFR.

Given the multiplicity of miRNAs, it seems quite clear that there are no definitive targets and that significant effort is still required.

3.3 MIRNA LIQUID TARGETS

Now Hoey and Liu present some detailed Liquid Biopsy targets as shown below:

Target	***Source***	***Use***	***Details***
let-7a	whole blood	Diagnostic prognostic	Decrease in PCa vs BPH; decreased expression with increasing D'Amico risk stratification
miR-9	Serum	Prognostic	increase in patients with metastatic PCa
miR-15b	Plasma	Prognostic	increased expression associated with high PSA after RP
miR-16	Plasma	Diagnostic	increased expression associated with high-grade (Gleason 8–10) disease
miR-20a	Plasma	Prognostic	increase in high-risk CAPRA score patients. Signature of miR-20a, miR-21, and miR-145 could distinguish patients with intermediate vs low-risk D'Amico scores. Signature of miR-20a, miR-21, miR-145, and miR-221 could distinguish patients with high- vs low risk D'Amico scores
miR-21	Plasma	Prognostic	increase in patients with high-risk CAPRA scores. increase in

Target	*Source*	*Use*	*Details*
			patients with intermediate- and high-risk vs low-risk D'Amico scores. Differentiate patients with intermediate- vs low-risk CAPRA scores. Signature of miR-20a, miR-21, and miR-145 could distinguish patients with intermediate- vs low-risk D'Amico scores. Signature of miR-20a, miR-21, miR-145, and miR-221 could distinguish patients with high- vs low-risk D'Amico scores
miR-24	Serum	Prognostic	Decrease in PCa: healthy > low risk > intermediate > high risk
miR-93	Serum	Diagnostic prognostic	increase in cancer vs healthy. Higher in high risk vs low and intermediate risk
miR-106a	Serum	Prognostic	increase in aggressive PCa
miR-106b	Plasma	Prognostic	increased expression associated with high PSA after RP
miR-130b	Plasma	Diagnostic	Part of miRNA signature to distinguish PCa and BPH
miR-135a	Serum	Prognostic	Decrease in aggressive PCa
miR-141	whole blood	Diagnostic prognostic	increase in PCa vs BPH. increased expression with increasing D'Amico risk stratification. Expression decreased after radical prostatectomy
miR-145	Plasma	Prognostic	increase in patients with intermediate- and high-risk vs low-risk D'Amico scores. Signature of miR-20a, miR-21, and miR-145 could distinguish patients with intermediate- vs low-risk D'Amico scores. Signature of miR-20a, miR-21, miR-145, and miR-221 could distinguish patients with high- vs low-risk D'Amico scores
miR-146a	Serum plasma	Predictive, prognostic	Low expression pre-docetaxel chemotherapy associated with PSA response in CRPC patients
miR-148a	Plasma	Diagnostic prognostic	increased expression associated with high PSA after RP and high- grade (Gleason 8–10) disease
miR-195	Plasma	Diagnostic	increased expression associated with high-grade (Gleason 8–10) disease
miR-200a	Serum plasma	Predictive, prognostic	High expression pre-docetaxel chemotherapy associated with decreased survival in CRPC patients
miR-200b	Serum plasma	Predictive, prognostic	High expression pre-docetaxel chemotherapy associated with decreased survival, PSA response in CRPC patients
miR-200c	Serum	prognostic Diagnostic	Decrease in aggressive PCa
miR-221	plasma Plasma	prognostic Prognostic	Differentiate patients with intermediate- vs low-risk CAPRA scores. Signature of miR-20a, miR-21, miR-145, and miR-221 could distinguish patients with high- vs low-risk D'Amico scores
miR-222	Serum	Predictive,	Low expression pre-docetaxel chemotherapy associated with PSA response in CRPC patients
miR-223	plasma Serum	prognostic Prognostic,	Decrease in cancer vs normal. increase in high risk vs low risk
miR-301b	Serum plasma	Predictive, prognostic	High expression pre-docetaxel chemotherapy associated with PSA response in CRPC patients
miR-375	Serum	Prognostic	increase in patients with metastatic PCa
miR-378	Serum	Prognostic	4 years after ADT failure and mortality at 20 months after ADT failure increase in high-risk and CRPC serum samples compared to low- risk localized ones. Decrease in low-risk localized and metastatic tumor specimens vs normal tissue
miR-429	Serum plasma	Prognostic, predictive	increase associated with decreased survival after docetaxel chemotherapy in CRPC patients
miR-433	Serum	Prognostic	increase in aggressive PCa
miR-451	Serum	Diagnostic prognostic	increase in high risk vs healthy; no change between healthy, low-, and intermediate-risk
miR-561a-3p	Serum	Prognostic	increase in patients with metastatic PCa
miR-605	Serum	Prognostic	Decrease in aggressive PCa

Target	*Source*	*Use*	*Details*
miR-1246		Prognostic	increased expression correlated with pathological grade, positive metastasis, poor prognosis, and tumor aggression in vitro and in vivo
miR-120	Plasma	Prognostic	increased expression associated with shorter overall survival at 4 years after ADT failure and mortality after ADT failure

It should be clear from the details above that there is a highly complex yet uncertain set of miRNA markers. The challenge is to select the correct one. There is diagnostic and prognostic one.

3.4 MIRNA PERFORMANCE

There have been few studies analyzing the performance of these miRNA markers. In the paper by Koppers-Lalic et al:

In many cancer types, the expression and function of ~22 nucleotide-long microRNAs (miRNA) is deregulated. Mature miRNAs can be stably detected in extracellular vesicles (EVs) in biofluids, therefore they are considered to have great potential as biomarkers. In the present study, we investigated whether miRNAs have a distinct expression pattern in urine-EVs of prostate cancer (PCa) patients compared to control males.

By next generation sequencing, we determined the miRNA expression in a discovery cohort of 4 control men and 9 PCa patients. miRNAs were validated by using a stemloop RT-PCR in an independent cohort of 74 patients (26 control and 48 PCa-patients). Whereas standard mapping protocols identified > 10 PCa associated miRNAs in urinary EVs, miR-21, miR-375 and miR-204 failed to robustly discriminate for disease in a validation study with RT-PCR-detection of mature miRNA sequences. In contrast, we observed that miRNA isoforms (isomiRs) with 3′ end modifications were highly discriminatory between samples from control men and PCa patients.

Highly differentially expressed isomiRs of miR-21, miR-204 and miR-375 were subsequently validated in an independent group of 74 patients.

Receiver-operating characteristic analysis was performed to evaluate the diagnostic performance of ***three iso-miRs, resulting in a 72.9% sensitivity with a high (88%) specificity and an area under the curve (AUC) of 0.866. In comparison, prostate specific antigen had an AUC of 0.707 and measuring the mature form of these miRNAs yielded a lower 70.8% sensitivity and 72% specificity (AUC 0.766).*** *We propose that isomiRs may carry discriminatory information which is useful to generate stronger biomarkers.*

Thus despite the proliferation of targets we see little clinical improvement.

4 BLADDER

Bladder cancer can become a highly invasive malignancy. The bladder is a sack with epithelial cells on the inside surrounded by muscle cells. It can contract when needed. The epithelial cells are frequently the ones which become malignant and are the ones which can be removed. If they are the only malignant cells then in a sense BCa can be a chronic disease. However once it penetrates to the muscle the chances for survival drop dramatically even after a complete removal of the organ. Thus it is critical to have better non-invasive diagnostic and prognostic measures.

As we have been noting, micro RNAs are small 22 base pair RNAs which we have discussed at length elsewhere[7]. BCa emits many miRNAs in the urine and even into the blood stream and they can have both specificity and sensitivity. Details profiling of these is still lacking[8]. However, we examine some in detail. As Larrrea et al have noted:

According to the National Cancer Institute, a biomarker is defined as "a biological molecule found in blood, other body fluids, or tissues that are a sign of a normal or abnormal process, or of a condition or disease."

In cancer, they can be divided into three general categories:

diagnostic biomarkers, which are used for a differential diagnosis;

prognostic biomarkers, which can distinguish tumors with a good outcome from those with a bad outcome; and

predictive biomarkers, which are for assessing whether a treatment is likely to be effective for a particular patient or not.

An ideal biomarker should have a high specificity, sensitivity, and predictive power. miRNAs have a number of intrinsic characteristics that make them attractive as biomarkers.

Firstly, they are highly specific, and it has been shown that miRNA expression profiles differ between cancer types according to diagnosis and the developmental stage of the tumor, with a greater resolution than traditional gene expression analysis.

Secondly, unlike other RNA classes, miRNAs are remarkably stable and therefore can be robustly measured not only in biological fluids but also from routinely prepared formalin-fixed paraffin-embedded (FFPE) material.

[7] https://www.researchgate.net/publication/334429457_miRNAs_Genes_and_Cancer_Cytology

[8] https://www.researchgate.net/publication/325046881_PCa_mir34_p53_MET_and_Methylation

Thus the miRNAs are excellent markers. As we shall note, there are many observed but like many of these markers we have specificity and sensitivity issues.

Indeed, unlike other RNA species, miRNAs appear resistant to boiling, pH changes, repeated freeze-thawing cycles, and fragmentation by chemical or enzymes. It should be noted, however, that cfmiRNAs are not themselves intrinsically resilient to RNase or any other treatment; rather, they are protected by their lipidic or protein-based carrier. As a result of these characteristics, the use of cfmiRNAs as biomarkers—and in particular as cancer biomarkers—has generated a plethora of publications over the last few years.

Due to the limitations of space, we will not attempt to review all of these but instead discuss the more robust studies that identify common cfmiRNA biomarkers in multiple studies. More often than not, these biomarker miRNAs are themselves intimately involved in cancer pathology,…, which includes their respective experimentally validated targets. While it may be tempting to speculate that these miRNAs may have the same effect while in circulation as intracellularly, there is no evidence that this is indeed the case.

4.1 SPECIFIC MIRNA TARGETS

The authors continue with the following table;

miRNA	***Cancer***	***Type Biomarker***	***Body Fluid***
miR-19a	Bladder	D	Plasma
miR-106b	Bladder	D	Urine
miR-210	Bladder	D, PG D	Serum Urine

Liu et al have also presented a table of other miRNAs as shown below, which seems quite extensive and frankly a bit unwieldly:

miRNA	Regulation	Source
miR-21	up	urine & BC cells lines
miR-200c	up	urine
miR-23b	up	urine
miR-513b-5p	up	urine
miR-183	up	urine
miR-205	up	from IBC patients
miR-16-1-3p, miR-28-5p, miR-92a-2-5p, miR-142-3p,		
miR-195-3p, miR-196b-5p, miR-299-3p, miR-492, miR-601, miR-619-5p, miR-3155a, miR-3162-5p, miR-3678-3p, miR-4283,	up	urine
miR-4295, miR-4311, miR-4531, miR-5096, miR-5187-5p		
miR-155-5p, miR-132-3p, miR-31-5p, miR-15a-5p	up	urine
miR-93, miR-940	up	urine
miR-16, miR-96	up	urine
miR-1, miR-99a, miR-125b, miR-133b, miR-143, miR-1207-5p	down	urine
let-7f-2-3p, miR-520c-3p, miR-4783-5p	down	urine
miR-30c-2-5p, miR-30a-5p	down	urine
miR-30a-5p, miR-30c-2-5p, miR-10b-5p	down	urine
miR-30a-5p, let-7c-5p	down	urine
miR-27b-3p	down	BC cells
miR-let-7i-3p	down	BC cells
miR-29c-5p, miR-146b-5p, miR-200a-3p, miR-200b-3p, miR-141-3p	down	BC cells

From Enokida et al we have the following list of miRNAs and their gene targets:

miRNA	***Type***	***Target Gene***
miR-1	TS	LASP1, PNP, PTMA, SRSF9, TAFLN2
miR-16	TS	CCND1
miR-23b	TS	EGFR, MET, ZEB1
miR-24	TS	CARD10, FOXM1
miR-27a	TS	RUNX1, SLC7A11
miR-27b	TS	DROSHA, EGFR, MET
miR-29c	TS	CDK6
miR-30a	TS	NOTCH1
miR-34a	TS	CD44, HNF4G, NOTCH1
miR-99a	TS	FGFR3
miR-100	TS	MTOR
miR-101	TS	COX2, MET, VEGFC
miR-124-3p	TS	ROCK1, CDK4
miR-125b	TS	E2F3, MMP13, SPHK1
miR-128	TS	VEGFC
miR-129	TS	GALNT1, SOX4
miR-133a	TS	EGFR, FSCN1, GSTP1, LASP1, PNP, PTMA, TAGLN2
miR-133b	TS	AKT1, BCL2L2, EGFR

miRNA	*Type*	*Target Gene*
miR-135a	TS	FOXO1
miR-138	TS	ZEB2
miR-143	TS	SERPIN1, AKT
miR-144-5p/3p	TS	CCNE1, CCNE2, CDC25A, PKMYT1
miR-145	TS	CBFB, CLINT1, FSCN1, ILK, PAK1, PPP3CA, SERPIN1, SOCS7, IGF1R
miR-186	TS	HMGN5
miR-193a-3p	TS	LOXL4, PSEN1, HOXC9
miR-195	TS	BIRC5, CDC42, CDK4, GLUT3, WNT7A
miR-200b	TS	MMP16
miR-200c	TS	BMI1, E2F3
miR-203	TS	BCL2L2, BIRC5
miR-214	TS	PDRG1
miR-218	TS	BMI1, LASP1
miR-221	TS	STMN1
miR-320a	TS	ITGB3
miR-320c	TS	CDK6
miR-449a	TS	CDC25A
miR-485-5p	TS	HMGA2
miR-490-5p	TS	FOS
miR-493	TS	FZD4, RHOC
miR-497	TS	BIRC5, WNT7A
miR-574-3p	TS	MESDC1
miR-576-3p	TS	CCND1
miR-590-3p	TS	TFAM
miR-1182	TS	TERT
miR-9	Onco	CBX7, CERS2
miR-10b	Onco	HOXD10, KLF4
miR-19a	Onco	PTEN
miR-96	Onco	CDKN1A
miR-150	Onco	PDCD4
miR-155	Onco	DMTF1
miR-182-5p	Onco	RECK, SMAD4
miR-708	Onco	CASP2

Thus, there is a large multiplicity and an even larger targeting set. Profiling of these in the context of what is a significant marker and not is yet to be done.

miRNAs have great potential. We have examined them previously but the need to find good targets is still and open question.

4.2 EZH2 AND MIRNA

A recent result demonstrated the interaction between miRNAs and controlling genetic products. Martínez-Fernández et al, have noted:

Several miRNAs have been demonstrated to be involved in EZH2 regulation. In Figure 2, we show a schematic representation of those miRNAs reported to interact with EZH2 in cancer development. Some of them directly regulated EZH2 post-transcriptionally, such rs miR-101, miR-26o, miR-214, miR-217, miR-124, miR-138, miR-98, miR-25, miR-30d, miR-199a, miR-29, miR-144 and Let 7 family. Among them, miR-101 has been found as negative regulator of EZH2 expression in BC. The miR-101-EZH2 axis stems from previous work in mouse fib-oblasts showing that during; senescence EZH2 downregulation together with the histone demethylase KDM2B induchy the expression of miR-101.

In this system, the enforced expression of KDM2B caused the demethylation of H3K36 repressing fhe expression of miRNAs let-7b and miR-101, which in turn increased the EZH2 expression, contributing to cell immortalization. In BC cells, a similar axit involving NDY1/KDM2B-miR-101-EZH2 has been identified EZH2 is not only regulated by, but it also regulates a wide variety of miRNAs through epigenetic repression.

These miRNAs may act as tumor suppressors, modulating tumor growth cancer stem cell phenotype, and cancer cell invasiveness. This EZH2-mediated repression has been demonstrated for miR-31, miR-200a-b-c, miR-181, miR-203, and Let 7b-c. Among them, miR-181a-b, miR-200a-b-c and miR-203 are regulated by EZH2 and inhibit the expression of BMI1 and RING2, both PRC1 members. These findings suggest a possible regulatory axis including miRNAs-EZH2-miRNAs-PRC1 in advanced cancer.

The situation of miR-200 family members in this axis is of particular relevance… that enforced expression of miR-200c in BC cell lines drastically reduced transcription factor E2F3, which acts as a positive activator of EZH2 and BMI1 transcription. Interestingly, the two miR-200 clusters are concurrently silenced by promoter hypermethylation in advanced BC.

In addition, increased EZH2, which is a common hallmark of NMIBC at high risk of recurrence and tumor progression in recurrences, also caused a decrease of miR-200 family expression, and the knock down of EZH2 or its inhibition, using DZNep (3-Deazaneplanocin A), resulted in an increased expression of the miR-200 family in BC cell lines. A similar effect of BMI1 suppressing miR-200 expression has been reported in other tumor types, such as breast and prostate and in BCa.

4.3 MTOR AND MIRNA

We have examined mTOR extensively. However miRNAs specifically miR100 has a strong role it plays. As Xu et al note:

miRNAs are involved in cancer development and progression, acting as tumor suppressors or oncogenes. In this study, miRNA profiling was conducted on 10 paired bladder cancer tissues using 20 GeneChip miRNA Array, and 10 differentially expressed miRNAs were identified in bladder cancer and adjacent noncancerous tissues of any disease stage/grade. After being validated on expanded cohort of 67 paired bladder cancer tissues and 10 human bladder cancer cell lines by quantitative real-time PCR (qRT-PCR), it was found that miR-100 was downregulated most significantly in cancer tissues.

Ectopic restoration of miR-100 expression in bladder cancer cells suppressed cell proliferation and motility, induced cell-cycle arrest in vitro, and inhibited tumorigenesis in vivo both in subcutaneous and in intravesical passage. Bioinformatic analysis showed that the mTOR gene was a direct target of miR-100. siRNA-mediated mTOR knockdown phenocopied the effect of miR-100 in bladder cancer cell lines.

In addition, the cancerous metastatic nude mouse model established on the basis of primary bladder cancer cell lines suggested that miR-100/mTOR regulated cell motility and was associated with tumor metastasis. Both mTOR and p70S6K (downstream messenger) presented higher expression levels in distant metastatic foci such as in liver and kidney metastases than in primary tumor.

Taken together, miR-100 may act as a tumor suppressor in bladder cancer, and reintroduction of this mature miRNA into tumor tissue may prove to be a therapeutic strategy by reducing the expression of target genes

This is just another example of the interactions between miRNAs and major control elements.

4.4 PROGNOSIS

Various studies have also focused on prognostic markers. As Enokida et al have noted:

Many retrospective studies have suggested that the expression of specific miRNAs in BCa specimens may be a good prognostic marker predicting overall survival, disease free survival, recurrence free survival, or progression free survival, ….

Moreover, several reports analyzing urine and blood samples have shown that miR- 200, miR-214, and miR-3187-3p levels are good prognostic markers in the blood.

Surprisingly, miR-203, miR- 214, miR-152, and miR-3187-3p were shown to be independent markers predicting overall survival, progression free survival, or recurrence free survival in multivariate studies. Unfortunately, no prospective studies have been performed to date. Thus, additional large, prospective studies are needed for the clinical application of miRNA assays for diagnostic and prognostic purposes in BC.

5 THYROID

The thyroid is an endocrine gland and it may develop various types of malignancies. There are papillary, follicular, both being somewhat indolent and then anaplastic, which is one of the most aggressive cancers if not the most aggressive. Anaplastic thyroid cancer can result in death in less than three months. It is rare and generally is a sequella to thyroid diseases. Thyroid cancers are the fastest growing in terms of incidence but mortality has decreased. One suspects that the increase in incidence is due to early detection of what would in other malignancies be termed carcinoma in situ.

Exosomes are small vesicles that contain DNA fragments, RNA fragments, miRNA, proteins and other materials ejected from a cell. We give a graphic below. They are small, 10-90 nm in diameter, they are cell like with a shell and material inside, and somehow, they can be directed, namely they may have receptors or ligands which can sense what to attach to.

Cells are dynamic entities and they collect material from their environment and then expel materials back into the environment. Cells may multiply, die off, grow and change. Some of the materials which cells throw off are proteins, DNA and fragments of them, RNA and fragments and miRNAs. Some of the materials are encapsulated in vesicles termed exosomes. The exosome is of most interest because its encapsulation both protects its content as well as presents markers for identifying it. The exosome is small, possibly 10 nm or larger.

The exosomes carry various elements around the body and can attach to other cells and transfer their content. Unlike autophagy, they do not digest their contents and unlike many other such things floating about they seem to avoid the attack by the immune system.

These vesicles can convey information, albeit often location independent, and they can also provide communications to other cells, often targeted cells. What do the exosomes resemble? Are they fully cell like, and do they have ligands and receptors? Receptors may be non-functional unless they do more than just transport their contents. Ligands however would be useful for targeting target cells to deposit their contents. Also how do they manage to deposit the contents when they may attach to a target cell? As Maas et al note:

The topology of EVs is similar to cells, with extracellular receptors and ligands positioned on the outside, and cytoplasmic proteins and RNAs on the inside. Thus, in order for EVs to functionally communicate with cells, different types of interactions may be involved.

This could include release of EV contents in the extracellular space, EV binding to the cell surface, EV-plasma membrane fusion, and uptake by endocytosis. For stimulation of cell signaling by EV-associated extracellular ligands, EVs may directly interact with cognate receptors located on the plasma membrane of cells (or vice versa). This recognition may also serve as a means of "addressing" EVs to certain cell types.

Such ligand-receptor interactions likely accounts for many targeted biological effects of EVs, including those caused by EV-carried growth factors, angiogenic factors and extracellular matrix (ECM) proteins. For delivery of RNAs or cytoplasmic proteins, EVs must not only bind to,

but also release their contents into recipient cells, either by direct fusion with the plasma membrane or with the endosomal membrane after endocytosis.

The discussion above regarding the ligand receptors comes up frequently and may be a significant factor when examining the targeting of the exosome. One interesting question may be; does the exosome also take up other materials in its journey? Also, in its intra and extra-vasation, does it act like a neutrophil sensing where to exit, or is it just happenstance? The who issue of exosome interaction is just commencing but will be critical to its understanding.

This is still a complex and poorly defined process but understanding it will be critical for therapeutic uses.

5.1 MIRNAS AND THYROID CANCER

miRNAs have been noted as both biomarkers in cells as well as extra-cellular environments. As Xia has noted, the following is a listing of some of the significant miRNAs in thyroid cancers:

miRNA	***Genes***	***Function***
miR-138		Loss of expression
miR-21, miR-31, miR-221, miR-222, miR-181b		dys-regulation
miR-30a, miR-125b, miR-26a		Down Regulation
miR-197, miR-346		Follicular thyroid cancer
miR-221, miR-222	p27Kip	Regulate cell cycle

The following miRNAs putatively regulate the related cancer stem cells (CSCs).

miRNA	***Function***
miR-290	Controls *de novo* DNA methylation through regulation of transcriptional repressors in mESs
miR-21	As a REST-regulated miRNA, suppresses the self-renewal of mESs, corresponding to the decreased expression of Oct4,Nanog, Sox2, and c-Myc
miR-24	TGF-regulated miR-24 promotes skeletal muscle differentiation
miR-203	Targets p63 to promote epidermal differentiation by restricting cell proliferation and inducing cell cycle
miR-155	Sustained expression in HSCs causes a myeloproliferative disorder [213]let-7 Regulates BT-IC stem cell-like properties by silencing more than one target
miR-125b	Inhibits osteoblastic differentiation by down-regulation of cell proliferation
miR-26a	Modulates osteogenic differentiation of human adipose tissue-derived stem cells by targeting SMAD1
miR-129	miR-520h targets ABCG2 and miR-129 targets EIF2C3 and CAMTA1 important in the development of HSCs
miR-134	Modulates the differentiation of mESs and causes post-transcriptional attenuation of Nanog and LRH1
miR-130a, miR-206	Regulate synthesis of the neurotransmitter substance P in human mesenchymal stem cell-derived neuronal cells
miR-17-92	cluster Promotes cell proliferation and inhibits differentiation of lung epithelial progenitor cells miR-17-5p targets Rbl2
miR-1-2	Regulates cardiac morphogenesis, electrical conduction, and cell cycle
miR-124	Targets laminin and integrin related to neuronal differentiation
miR-1, miR-133, miR-206	Regulate the expression of the alternative splicing factor nPTB during muscle development
miR-181	Targets the homeobox protein Hox-A11 during mammalian myoblast differentiation
miR-9, miR-124a	Modulate embryonic stem cell-derived neurogenesis

Now as Pardini and Calin have noted:

Hormones are messengers circulating in the body that interact with specific receptors on the cell membrane or inside the cells and regulate, at a distal site, the activities of specific target organs. The definition of hormone has evolved in the last years. Hormones are considered in the context of cell–cell communication and mechanisms of cellular signaling.

The best-known mechanisms of this kind are chemical receptor-mediated events, the cell–cell direct interactions through synapses, and, more recently, the extracellular vesicle (EV) transfer between cells. Recently, it has been extensively demonstrated that EVs are used as a way of communication between cells and that they are transporters of specific messenger signals including non-coding RNAs (ncRNAs) such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs). Circulating ncRNAs in body fluids and extracellular fluid compartments may have endocrine hormone-like effects because they can act at a distance from secreting cells with widespread consequences within the recipient cells. Here, we discuss and report examples of the potential role of miRNAs and lncRNAs as mediator for intercellular communication with a hormone-like mechanism in cancer …

It has been demonstrated that miRNAs can regulate directly genes encoding hormones or other enzymes involved in hormone maturation and metabolism. miRNAs can also

target hormone antagonists or receptors indirectly modifying the hormone-mediated cell signaling transmission or could be regulated by hormones either at the level of miRNA transcription and processing. For instance, miR-21 and miR-181-b1 genes are expressed after STAT3 induction, which is activated by interleukin 6 (IL-6). Moreover, miR-21 is repressed by thyroid hormone (TH) and this downregulation regulates GRHL3, a transcriptional inhibitor of type 3 iodothyronine deiodinase (D3) which, in turn, inactivates TH.

5.2 DETAILED TARGETS

Now more specific markers have been noted for the different thyroid cancers. Yoruker et al have noted:

Papillary thyroid cancer is the most common form of thyroid cancer. However, reliable molecular markers with clinical and prognostic utility are not available for PTC. Recent studies indicate that miRNAs can exert oncogenic and tumor suppressor functions by modulating key pathways during tumor formation and progression. Several miRNA molecules have been associated with tumor proliferation, invasion or metastasis indicating that miRNAs may also act as predictive biomarkers in cancer.

The impact of miRNAs on cell growth, proliferation and metastasis has been noted and commented on by many authors. It is not clear which miRNAs are involved, although many have been suggested, and moreover it is still unclear as to the overall set of processes involved. We understand many of the cellular pathways regarding genes and their RNA and protein products. We understand upstream and downstream events. However this understanding is lacking for miRNAs in general.

Circulating miRNAs are ideal markers for the non-invasive detection of cancer since miRNA expression is dysregulated in cancer and is usually tumor-specific, displaying a consistent pattern. However, it is still unclear whether circulating miRNA levels may actually help in the differential diagnosis or can predict prognosis of PTC. In this study we analyzed a panel of miRNAs in terms of their utility in the differential diagnosis of thyroid cancer and their association with the clinical parameters as potential biomarkers for the disease. miR-21 has been reported to aid in distinguishing the papillary tumor variants in a selected miRNA panel.

Circulating miRNA, in this case miR-21 can be a putative marker but clearly not dispositive of PTC.

In our study circulating miR-21 levels were downregulated both in the patients with PTC and benign thyroid disease compared to the healthy individuals. In an earlier study higher levels of miR-21 have been reported in tumor tissue when compared to normal tissue. It has also been suggested that higher miR-21 expression levels are detected in PTC tissue samples when compared to MG. This was not the case in our study. However, these previous studies comprised nine and ten patients, respectively and our sample size is three times greater. Although upregulation of miR-21 in the patients has been reported in some studies other reports failed to confirm this and decreased expression in the plasma has also been reported in subgroups of tumors.

Furthermore, our data is in accordance with a very recent report which has found significantly decreased miR-21 levels in papillary thyroid cancer . miR-221 and miR-222 are overexpressed in PTC and have been associated with aggressive tumors and adverse clinical characteristics.

High levels of miR-146b and miR-222 have been reported in aggressive papillary thyroid cancer.

Prognosis is a key interest in thyroid malignancies. In a sense, like PCa, thyroid cancer, TC, is generally slow growing and if excised early can be curative. However, it is essential to be able to identify the aggressive forms. Noted above is a set of miRNAs which are suggestive. We do, however, have to ask, why these and what are their functions.

Evaluation of the pre- and postoperative groups showed that miR-221 and miR-222 levels were significantly lower after surgery. A significant decline was also observed following resection of the tumors in patients with Stage III/IV disease for miR-222. Combined with the fact that preoperative miR-221 levels are higher in tumors that are smaller than 2 cm when compared to larger tumors this ***may indicate that miR-221 and miR-222 expression levels in the circulation may be associated with the tumor burden****. This conclusion is in accordance with previous studies and with a report suggesting that miR-222 is important for detecting recurrence in PTC. While postoperative miR-222 levels were lower for all tumors a significant decline of the postoperative miR-221 levels was observed only in tumors smaller than 2 cm.*

TC is monitored after complete thyroidectomy by measuring thyroglobulin (TG), a protein in the thyroid cells which in response to TSH transports iodine into the follicle where it is used to enzymatically create T3 and T4. TG is produced only in thyroid cells and thus removing the thyroid should eliminate TG. However if the lesion has metastasized then TG is produced by the TG mets as they seek other places in the body. Can this be said for the miRNAs and how do the miRNAs relate to TG.

These findings support previous reports suggesting that miR-221 is one of the most sensitive miRNA molecules for thyroid cancer. Microarray data suggest that miR-146b is overexpressed in many cancer types, such as melanoma, lung and colorectal cancer. The miR-146b molecule plays an important role in tumor progression by downregulating BRCA1. We observed higher postoperative miR-146b levels in patients with tumors larger than 2 cm. This is in line with studies suggesting that miR-146b levels in fine needle aspiration biopsies may help in detecting and identifying thyroid cancer.

Our data support the observations that miR-146b is associated with the risk of recurrence and may promote cell migration and invasion. This finding is also consistent with the fact that an association with tumor size has been shown for several miRNA molecules.

miR-31 and let7e molecules have been characterized as tumor suppressors in different cancer types. Deregulation of let-7 is observed in different types of cancer.

Let-7 genes are known to be highly expressed in the thyroid gland and let-7 downregulation has been associated with thyroid tumors. Serum miR-31 expression levels were not different between the disease and the control groups. A study has suggested that higher miR-31 expression is

observed in tissue biopsy samples from oncocytic adenomas. In our study neither the PTC nor the MG samples displayed aberrant miR-31 expression. This is in concordance with a recent study which has not found differential expression of miR-31 between the normal tissue and thyroid tumors. Although much higher let-7 levels were observed in the patients with PTC the difference was not significant possibly due to the wide distribution of the values. However, discrepant expression of let-7 is also consistent with a previous study. miR-151-5p has been implicated in several pathological conditions as well as cancer, and was found to be correlated with the migration and invasion of cancer cells.

The targets for TG miRNA keep expanding. It is clear that they have significant potential and should be explored. Finally:

*Evaluation of the pre and postoperative groups **showed significantly lower miR-151-5p levels in postoperative patients with late stage tumors.** This is in concordance with a previous study reporting a significant decrease of the miR-151-5p levels following tumor excision and is also in line with the suggestion that miR-151-5p is associated with lymph node metastasis and larger tumor size.*

Additional miRNAs have been listed by Martini et al.

5.3 BIOMARKER

Let us now consider some comments regarding biomarker options. As Rappa et al have noted:

Recent results in other types of cancer, described in the paragraph above, suggest that information derived from analysis of EVs from peripheral blood plasma can be integrated in the routine diagnostic approach to the patient with non-functional thyroid nodules. Moreover, specific alterations of cellular miRNA expression profile have been reported in thyroid carcinoma, indicating the possibility that some of these miRNAs, contained in EVs, may be employed as circulating biomarkers. miRNAs in the circulation have been analyzed as potential biomarkers of recurrence in PTC.

In many cases in which serum thyroglobulin measurements are difficult to interpret, the analysis of changes in circulating levels of miR-146a-5p and miR-221-3p in PTC patients indicate a good correlation with the American Thyroid Association (ATA)-defined response to therapy classes.

*…serum levels of **miR-146a-5p and miR-221-3p** could be used as complementary biomarkers for the early non-invasive detection of persistent PTC. The association between high circulating levels of miR-146b, miR-222, miR-221, and follicular thyroid proliferation has recently been described.*

*Two miRNAs (**miR-95, miR-190**) were differently expressed in serum of PTC patients. In particular, miR-190 was up-regulated whereas miR-95 was down-regulated, which in combination can be used for the differential diagnosis of thyroid nodules.*

Other studies have shown that the circulating levels of ***miR-146b-5p, miR-221-3p, miR-222-3p, and miR-146a-5p*** *were reduced upon tumor excision. The up-regulated expression of miR-146b-5p, miR-221-3p, and miR-222-3p in the circulation of patients with thyroid cancer has also been demonstrated in PTC, as well as in anaplastic and follicular thyroid carcinoma*

Also, Samsonov et al. found that plasma exosomal ***miR-21 and miR-181****a differentiate follicular from PTC. An analytical approach employing a miRNA-based assay on thyroid fine needle aspirate smears from routinely prepared cytology slides has recently been proposed to improve the diagnostic process. In addition, we have recently proposed a new miRNA-based molecular classification of PTC*

The above distinction of follicular from papillary is useful. Papillary generally is the most indolent, yet the classification of follicular variant of papillary thyroid cancer, FVPTC, is an interesting such variant since the cells appear as in PTC but the papilla are missing and the growth pattern is follicular. This variant is often the most indolent. Yet monitoring it as noted previously is critical.

6 GASTROINTESTINAL

GI cancers are a broad mix of cancers ranging from the esophagus, stomach, small and large intestine and the liver and gall bladder. I exclude the pancreas since that is often an endocrine cancer. GI cancers are often hidden, except when examined in such a manner as a colonoscopy. Colon cancer can be slow growing and with colonoscopies the mortality can be reduced dramatically.

6.1 MIRNA MARKERS

As Shigeyasu et al noted:

The field of miRNA-based cancer research has witnessed a remarkable evolution over the last two decades. Although much effort to date has been to identify specific miRNAs and their role in cancer, interest has grown to evaluate their potential as disease biomarkers, as well as recent attempts at exploiting their significance as therapeutic targets.

Their small size and stability in a variety of body fluids make them attractive substrates for biomarker development. As this field continues to mature with the identification of more specific subtypes of miRNAs and increase in focus on large-scale, multicenter, comprehensive studies, miRNA based diagnostic approaches are likely to usher in a new era of personalized medicine for patients with cancer.

They include the following Table detailing targets for a variety of GI cancers.

miRNA	***Source***	***AUC***	***Sensitivity***	***Specificity***
Colorectal cancer Single marker				
miR-17-3p	Plasma	0.72	64	70
miR-92	Plasma	0.89	89	70
miR-21	Serum	0.93	83	91
miR-23a	Exosome	0.95	NA	NA
miR-378	Plasma	0.95	NA	NA
miR-1246	Exosome	0.95	NA	NA
Panel				
miR-431, 15b, 139-3p	Plasma	0.83	91	57
miR-532-3p, 331, 195, 17, 142-3p, 15b, 532, 652	Plasma	0.87	88	64
miR-19a-3p, 223-3p, 92a-3p, 422a	Serum	0.95	84	92
miR-19a-3p, 223-3p, 92a-3p, 422a	Serum	0.77	NA	NA
miR-21, 31, 92a, 181b, 203, let-7g	Serum	0.92	96	88
miR-21, 29a, 125b	Serum	0.83	NA	NA
Esophageal cancer				
Single marker				
miR-18a	Plasma	0.94	87	100
miR-1246	Serum	0.75	71	74

miRNA	*Source*	*AUC*	*Sensitivity*	*Specificity*
miR-25	Plasma	0.86	85	86
Panel				
miR-10a, 22, 100, 148b, 223, 133a, 127-3p	Serum	0.93	79	96
miR-21/375 (ratio)	Plasma	0.82	88	70
miR-25, 100, 193-3p, 194, 223, 337-5p, 483-5p	Serum	0.83	81	81
Gastric cancer				
Single marker				
miR-16	Serum	0.90	79	78
miR-18a	Plasma	0.91	81	85
miR-222	Plasma	0.85	66	88
miR-21	Serum	0.91	88	80
Panel				
miR-106a/let-7a (ratio)	Plasma	0.88	86	80
miR-1, 20a, 27a, 34, 423-5p	Serum	0.88	80	81
miR-223, 21, 218	Plasma	0.95	84	93
Hepatocellular carcinoma				
Single marker				
miR-21	Serum	0.87	84	74
miR-122	Serum	0.79	71	69
miR-223	Serum	0.86	80	77
miR-18a	Serum	0.88	86	75
Panel				
miR-122, 192, 21, 223, 26a, 27a, 801	Plasma	0.94	83	94
miR-375, 25, let-7f	Serum	0.99	98	99
miR-23b, 423, 375, 23a, 342-3p	Serum	0.99	97	99
miR-29a, 29c, 133a, 143, 145, 192, 505	Serum	0.82	75	89
Pancreatic cancer				
Single marker				
miR-200a	Serum	0.86	84	88
miR-200b	Serum	0.85	71	97
miR-27a-3p	Whole blood	0.86	82	79
miR-1290	Serum	0.96	88	84
Panel				
miR-16, 196a (with CA19-9)	Plasma	0.98	92	96
miR-20a, 21, 24, 25, 99a, 185, 191	Serum	0.99	94	93
miR-145, 150, 223, 636	Whole blood	0.83	85	48
miR-26b, 34a, 122, 126^{m}, 145, 150, 223, 505, 636, 885-5p	Whole blood	0.82	85	55
Biliary cancer				

miRNA	*Source*	*AUC*	*Sensitivity*	*Specificity*
Single marker				
miR-21	Plasma	0.93	85	100
miR-126	Serum	0.87	68	93
miR-1281	Serum	0.83	55	90
Panel				
miR-6075, 4294, 6880-5p, 6799-5p, 125a-3p, 4530, 6836-3p, 4476	Serum	0.95	80	98

6.2 BIOMARKER

Genetic biomarkers have been delivered for colon cancer. Moody et al have noted:

In 2014, the multitarget DNA test Cologuard® was FDA-approved and made commercially available in the USA. In addition to fecal hemoglobin, Cologuard® assays DNA methylation in the bone morphogenetic protein 3 (BMP3) and NDRG family member 4 (NDRG4) promoters and mutations in the KRAS proto-oncogene (KRAS) gene.

The assay was reported to detect CRC with a sensitivity of 92% and premalignant lesions with a sensitivity of 42%. In a screening of over 400 asymptomatic adults, methylation of BMP3 showed greater specificity for polyp detection than the FIT . Another study in over 1000 subjects found that Cologuard® detected CRC with 90% specificity and 98% sensitivity . It was further noted that Cologuard® could detect precancerous lesions with a sensitivity of 57% for precursors ≥ 1 cm and 83% for precursors > 3 cm.

This evidence suggests that fecal genetic markers can provide a viable means of cancer detection…

Circulating miRNA may serve as a reflection of the underlying disease in CRC. Several plasma miRNA have been shown to be dysregulated in CRC. miR-92 was first reported as a possible noninvasive biomarker for CRC diagnosis in 2009. Since then, a recent meta-analysis of over 500 colorectal cancer patients reported that miR-92a had a diagnostic sensitivity of 76% and sensitivity of specificity of 64% . When several miRNAs (let-7g, miR-21, miR-92a, miR-181b, and miR-203) in serum were used as a biomarker profile panel for CRC diagnosis, sensitivity and specificity increased to 93 and 91%, respectively. The same serum samples showed only 35% sensitivity and 23% specificity for CRC when CEA and CA19-9 were used.

In addition to distinguishing normal individuals from cancer patients, circulating miRNA profiles have been shown to differ between healthy controls and patients with pre-cancerous adenomas. High-throughput sequencing has facilitated the discovery of many more circulating miRNA that are differentially expressed in CRC; however, no blood-based miRNA tests are currently being used to screen for CRC…

Moody also contains the following table.

miRNA	*Blood or stool*	*Sensitivity (%)*	*Specificity (%)*
miR-92a	Blood and stool	72–79 (blood) 72 (stool)	59–69 (blood) 73 (stool)
miR-20a	Blood and stool	46 (blood) 55 (stool)	73 (blood) 82 (stool)
miR-21	Blood and stool	62–85 (blood) 56 (stool)	79–88 (blood) 73 (stool)
miR-221	Blood and stool	86 (blood) 62 (stool)	41 (blood) 74 (stool)
miR-18a	Stool	61	69
miR-135b	Stool	78	68
miR-144*	Stool	74	87
miR-199a-3p	Blood	48	75
miR-155	Blood	58	95
miR-183	Blood	74	89
miR-29a	Blood	53–65	85–93
miR-29b	Blood	61	73
miR-210	Blood	75	74
miR-196b	Blood	63	87
miR-139-3p	Blood	97	98
miR-622	Blood	88	64
miR-506	Blood	61	77
miR-4316	Blood	75	77

7 MELANOMA

Melanoma is an aggressive and deadly cancer. Yet just ten years ago that picture began to change. We have seen many successes in the use of immunotherapy and the like to improve survival and quality of life. Yet there are still cases which cannot be reached. We have previously written extensively about miRNA and melanoma[9]. We build on those previous insights.

Forloni et al had noted:

Oncogenic mutations in BRAF and NRAS occur in 70% of melanomas. In this study, we identify a microRNA, miR-146a, that is highly upregulated by oncogenic BRAF and NRAS. Expression of miR-146a increases the ability of human melanoma cells to proliferate in culture and form tumors in mice, whereas knockdown of miR-146a has the opposite effects. We show these oncogenic activities are due to miR-146a targeting the NUMB mRNA, a repressor of Notch signaling.

Previous studies have shown that pre-miR-146a contains a single nucleotide polymorphism (C>G rs2910164). We find that the ability of pre-miR-146a/G to activate Notch signaling and promote oncogenesis is substantially higher than that of pre-miR-146a/C. Analysis of melanoma cell lines and matched patient samples indicates that during melanoma progression pre-miR-146a/G is enriched relative to pre-miR-146a/C, resulting from a C-to-G somatic mutation in pre-miR-146a/C. Collectively, our results reveal a central role for miR-146a in the initiation and progression of melanoma

In a recent paper by Reuland et al the authors make the following observations[10]:

Melanoma is an aggressive cancer that metastasizes rapidly and is refractory to conventional chemotherapies. Identifying microRNAs (miRNAs) that are responsible for this pathogenesis is therefore a promising means of developing new therapies. We identified miR-26a through microarray and quantitative reverse-transcription–PCR (qRT-PCR) experiments as a miRNA that is strongly downregulated in melanoma cell lines as compared with primary melanocytes. Treatment of cell lines with miR-26a mimic caused significant and rapid cell death compared with a negative control in most melanoma cell lines tested.

In surveying targets of miR-26a, we found that protein levels of SMAD1 (mothers against decapentaplegic homolog 1) and BAG-4/SODD were strongly decreased in sensitive cells treated with miR-26a mimic as compared with the control.

[9] https://www.researchgate.net/publication/325106051_mi_RNA_and_Melanoma also https://www.researchgate.net/publication/325046958_NOTCH_PATHWAY_MIR-146A_AND_MELANOMA

[10] http://www.nature.com/jid/journal/vaop/ncurrent/full/jid2012400a.html

The luciferase reporter assays further demonstrated that miR-26a can repress gene expression through the binding site in the 3' untranslated region (3'UTR) of SODD (silencer of death domains). Knockdown of these proteins with small interfering RNA (siRNA) showed that SODD has an important role in protecting melanoma cells from apoptosis in most cell lines sensitive to miR-26a, whereas SMAD1 may have a minor role. Furthermore, transfecting cells with a miR-26a inhibitor increased SODD expression. Our findings indicate that miR-26a replacement is a potential therapeutic strategy for metastatic melanoma, and that SODD, in particular, is a potentially useful therapeutic target.

The observations focus on several key areas:

1. The impact of miRNAs on melanoma metastasis. As we will discuss there have been many previous studies implicating many miRNAs in this area. Thus seems to expand the results.

2. There appears to be a therapeutic approach to the issue by increasing the miRNA26a to further reduce by binding to the SODD facilitator product. There again has been several studies along this line recently.

3. There may be a prognostic indicator here as well. Again there has been a great deal of work in this field.

First we examine both the miRNA26a and SODD respectively and then we examine the issues discussed above in some detail. This represents just another of many studies regarding the use of miRNAs for the potential control of melanoma.

Now a press release states[11]:

A University of Colorado Cancer Center study in this month's edition of the Journal of Investigative Dermatology describes a new target and potential treatment for melanoma, the most dangerous form of skin cancer. MicroRNA can decide which genes in a cell's DNA are expressed and which stay silent. Melanoma tends to lack microRNA-26a, which makes the gene SODD go silent.

"It's a double negative," says Yiqun Shellman, PhD, investigator at the CU Cancer Center, associate professor at the CU School of Medicine, and the study's co-senior author. "miR-26a works to stop the growth of cancer. You turn off this thing that should stop growth, and you have growth." When Shellman, David Norris and colleagues reintroduced microRNA-26a to melanoma cell lines that lacked it, they saw a marked decrease in cancer cell survival. MicroRNA-26a killed melanoma cells while leaving healthy cells unharmed. In fact, the discovery started back a couple steps.

[11] http://medicalxpress.com/news/2012-12-serendipity-potential-therapy-melanoma.html also http://medicalxpress.com/news/2012-12-serendipity-potential-therapy-melanoma.html#jCp

First the group compared microRNA expression in healthy cells to that of microRNA expression in melanoma cells. "We hoped the difference between microRNA expression in healthy and melanoma cells would show which ones were contributing to tumorgenesis," Shellman says.

The microRNA most consistently different between healthy and cancerous cells was 26a. The discovery of how it works and what exactly it does was serendipitous. "We started by testing the effect of microRNA-26a on known gene targets to see if it was effecting the expression of logical, cancer-causing pathways, but none of them seemed affected in melanoma," Shellman says.

"We were working with the SODD gene in an unrelated project, and SODD has a putative but not high-scored binding site for miR-26a, and thought, why not test it? Sure enough, it turned out to be the target – microRNA-26a downregulates this gene." Shellman hopes this robust finding in cell cultures will help pave the way for future work with microRNA-26a as a therapeutic target in animal models and eventually a human trial.

"The first step is to further pinpoint the genetic signatures of the patients likely to benefit from microRNA-26a replacement therapy," Shellman says, noting that only some and not all melanoma cells were killed by miRNA replacement. "Maybe it's simply the downregulation of microRNA-26a itself, or maybe we can use SODD expression as the biomarker," Shellman says.

Once Shellman and colleagues discover the characteristics of a melanoma susceptible to microRNA-26a treatment, they hope funding will allow the lab to follow the promising therapy up the evolution from cells to humans.

As can be seen from the conversation above there still may exist some questions of the details of the process. What is critical, however, is the fact that the miRNA plays such a prominent role, that one may target the miRNA, and that a pathway is a fundamental part of the development of a putative therapeutic. But fundamentally the last sentence above does diminish the ultimate enthusiasm.

Another Press review states as follows[12]:

Researchers from the University of Colorado Cancer Center say that they have discovered a new, more targeted way of treating melanoma, the most deadly form of skin cancer.
The findings, described in a recent edition of the Journal of Investigative Dermatology, describe how small pieces of genetic material known as MicroRNA can choose the genes in a DNA cell that are either expressed or kept silent. With melanoma in particular, the researchers discovered a deficiency of microRNA-26a that usually silences the gene SODD.

"It's a double negative," explained the study's co-senior author Yiqun Shellman, an investigator at the University of Colorado Cancer Center and associate professor at the University of

[12] http://www.redorbit.com/news/health/1112752907/genetic-culprit-for-melanoma-found-122112/

Colorado School of Medicine, in a prepared statement. "MiR-26a works to stop the growth of cancer. You turn off this thing that should stop growth, and you have growth."

In the study, melanoma cell lines that lacked microRNA-26a were reintroduced to the cell in a lab. As a result, there was a reduction in cancer cell survival and the microRNA-26a eliminated melanoma cells while leaving healthy cells alive. The team of investigators was able to compare the expression of microRNA in healthy cells to the expression of microRNA in melanoma cells.

"We hoped the difference between microRNA expression in healthy and melanoma cells would show which ones were contributing to tumorgenesis," continued Shellman in the statement. The researchers saw that the expression of micro-RNA-26 was consistently different between healthy and cancerous cells. Some, but not all, of the melanoma cells were eliminated by the replacement introduction of mRNA.

"The first step is to further pinpoint the genetic signatures of the patients likely to benefit from microRNA-26a replacement therapy," noted Shellman in the statement. "Maybe it's simply the downregulation of microRNA-26a itself, or maybe we can use SODD expression as the biomarker."

Moving forward, Shellman believes that her team's discovery of the role of MicroRNA in the development of carcinoma in cell cultures may eventually help develop new therapeutic techniques that could be used in real cancer patients.

This above statement is a simple reiteration of some of the prior work. Again it is clear that although experimentally observed, one is still quite a way from clinical reality.

7.1.1 SODD Pathway

Let us first consider the SODD pathway. From the Biocarta database we have the following[13].

The tumor necrosis factor (TNF) receptor superfamily contains several members with homologous cytoplasmic domains known as death domains (DD). The intracellular DD are important in initiating apoptosis and other signaling pathways following ligand binding by the receptors. In the absence of ligand, DD-containing receptors are maintained in an inactive state.

TNF RI contains a cytoplasmic DD required for signaling pathways associated with apoptosis and NF-kB activation… widely expressed 60 kDa protein, named SODD (silencer of death domains), associated with the DD of TNF RI and DR3. Overexpression of SODD suppresses TNF-induced cell death and NF-kB activation demonstrating its role as a negative regulatory protein for these signaling pathways.

TNF-induced receptor trimerization aggregates the DD of TNF RI and recruits the adapter protein TRADD.3,5 This in turn promotes the recruitment of the DD-containing cytoplasmic proteins FADD, TRAF2 and RIP to form an active TNF RI signaling complex. In contrast,

[13] http://www.biocarta.com/pathfiles/h_soddpathway.asp

SODD acts as a silencer of TNF RI signaling and does not interact with TRADD, FADD, or RIP. It is associated with the DD of TNF RI and maintains TNF RI in an inactive, monomeric state. TNF-induced aggregation of TNF RI promotes the disruption of the SODD-TNF RI complex.

SODD does not interact with the DD of other TNF receptor superfamily members such as Fas, DR4, DR5, or TNF RII. SODD association with TNF RI may represent a general model for the prevention of spontaneous TNF signaling by other DD-containing receptors.

In a recent paper by Viatour et al, the authors examine the specifics of the NF-kB pathway elements regarding inflammation and cancer. We take the figure below as modified from their work. It demonstrates the SODD impact as an integrated whole.

The classical (blue arrows), alternative (green arrows) and atypical (purple arrows) NF-κB-activating pathways as illustrated by the TNF-α-mediated, CD40-mediated and DNA-damage-mediated NF-κB activation pathways, respectively. In the classical NF-κB-activating pathway, upon binding of TNFα to TNFR1, SODD is released from the receptor and triggers the sequential recruitment of the adaptors TRADD, RIP and TRAF2 to the membrane.

Then, TRAF2 mediates the recruitment of the IKK complex – composed of IKKα, IKKβ and NEMO – to the TNFR1 signalling complex. Hsp90 and Cdc37 are also part of the IKK complex and are required for the TNFα-induced IKK activation and shuttling of the IKK complex from the cytoplasm to the membrane, and ELKS connects IκBα to the IKK complex .

Activation of the IKK complex leads to the phosphorylation of IκBα at specific residues, ubiquitination through binding of ubiquitin proteins and degradation of this inhibitory molecule via the proteasome pathway. Then, the heterodimer p50–p65 is released and migrates to the nucleus where it binds to specific κB sites and activates a variety of NF-κB target genes, including IL-8, IL-6, TNFα and many more. The alternative pathway is triggered by binding of the CD40 ligand to its receptor, leading to recruitment of TRAF proteins and the sequential activation of NIK and IKKα, which then induces the processing of the inhibitory protein p100. p100 proteolysis releases p52 which forms heterodimers with RelB. This pathway is NEMO-independent and relies on IKKα homodimers. The atypical pathway, which is triggered by DNA damage such as UV, relies on sequential p38 and CK2 activations, and involves phosphorylation and subsequent IκBα degradation via an IKK-independent pathway.

Subsequently, free NF-κB moves into the nucleus to activate its target genes. Note that the DNA-damaging agent doxorubicin also triggers p65 phosphorylation via a p53- and RSK1-dependent pathway (not shown). Phosphorylation of the signalling molecules in addition to NF-κB and IκB proteins are illustrated.

Abbreviations: CK2, casein kinase 2; ELKS, Glu-Leu-Lys-Ser; Hsp90, heat shock protein 90; IκB, inhibitor of NF-κB; IKK, IκB kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; NIK, NF-κB-inducing kinase; RIP, receptor-interacting protein; RSK1, ribosomal S6 kinase 1; SODD, silencer of death domains; TNF-α, tumour necrosis factor α; TNFR1, TNF receptor 1; TRADD, TNF-receptor-associated death domain protein; TRAF, TNF-receptor-associated factor; Ub, ubiquitin.

Tschopp et al discuss apoptosis as in which follows[14]:

Spontaneous signaling from death-domain-containing receptors can result in inappropriate cell death. An inhibitory protein has recently been identified, called silencer of death domains (SODD), that binds to the death domain of tumor necrosis factor receptor 1, thereby negatively regulating downstream signaling.

Members of the tumor necrosis factor (TNF) ligand family are critically involved in the regulation of infections, inflammation, autoimmune diseases and tissue homeostasis. Binding of these ligands to their respective receptors leads to the triggering of diverse signaling pathways, including the activation of caspases, the activation and nuclear translocation of nuclear factor κB (NF-κB), and the activation of mitogen-activated protein kinases such as Jun N-terminal kinase (JNK). As each of these pathways leads to different outcomes, TNF-related ligands can promote apoptosis, differentiation, or proliferation.

The TNF-related factors are synthesized as trimeric type II transmembrane molecules that are capable of oligomerizing (trimerizing) the corresponding receptor. Consequently, ligand-bound receptors transmit the signal across the membrane by bringing their cytoplasmic portions into close proximity, leading to the recruitment and activation of downstream effector proteins. Those members of the TNF receptor family that induce apoptosis have in their cytoplasmic region a sequence of approximately 70 amino acids called the death domain, which is essential for signal transmission.

These domains represent protein–protein interaction motifs that have an intrinsic propensity to oligomerize and to interact with other death domains in a homophilic fashion. At present, six death-domain-containing receptors have been identified — Fas, TNF receptor 1 (TNF-R1), death receptor 3 (DR3; also known as TRAMP, Wsl, APO-3 and LARD), the two receptors for TNF-related apoptosis-inducing ligand (TRAIL) TRAIL-R1/DR4 and TRAIL-R2/DR5, and DR6

Now from NCBI we have the definition and description of SODD (SODD (silencer of death domains) ; or BAG-4 8p11.23, BAG4 BCL2-associated athanogene) as follows[15]:

The protein encoded by this gene is a member of the BAG1-related protein family. BAG1 is an anti-apoptotic protein that functions through interactions with a variety of cell apoptosis and growth related proteins including BCL-2, Raf-protein kinase, steroid hormone receptors, growth factor receptors and members of the heat shock protein 70 kDa family. This protein contains a BAG domain near the C-terminus, which could bind and inhibit the chaperone activity of Hsc70/Hsp70. This protein was found to be associated with the death domain of tumor necrosis factor receptor type 1 (TNF-R1) and death receptor-3 (DR3), and thereby negatively regulates downstream cell death signaling. The regulatory role of this protein in cell death was demonstrated in epithelial cells which undergo apoptosis while integrin mediated matrix

[14] http://www.sciencedirect.com/science/article/pii/S0960982299802334

[15] http://www.ncbi.nlm.nih.gov/gene/9530

contacts are lost. Alternatively spliced transcript variants encoding distinct isoforms have been identified

Similarly for SMAD, NCBI states[16]:

SMAD1 SMAD family member 1 or BSP1; JV41; BSP-1; JV4-1; MADH1; MADR1 at 4q31

The protein encoded by this gene belongs to the SMAD, a family of proteins similar to the gene products of the Drosophila gene 'mothers against decapentaplegic' (Mad) and the C. elegans gene Sma. SMAD proteins are signal transducers and transcriptional modulators that mediate multiple signaling pathways. This protein mediates the signals of the bone morphogenetic proteins (BMPs), which are involved in a range of biological activities including cell growth, apoptosis, morphogenesis, development and immune responses.

In response to BMP ligands, this protein can be phosphorylated and activated by the BMP receptor kinase. The phosphorylated form of this protein forms a complex with SMAD4, which is important for its function in the transcription regulation. This protein is a target for SMAD-specific E3 ubiquitin ligases, such as SMURF1 and SMURF2, and undergoes ubiquitination and proteasome-mediated degradation. Alternatively spliced transcript variants encoding the same protein have been observed.

7.1.2 miRNA-26a in Other Cancers

miRNA26a has been found to play a role in other cancers. We examine a few here.

Dang et al state regarding miRNA 26a the following[17]:

MicroRNAs (miRNAs) are a class of 21-23 nucleotide RNA molecules that play critical roles in the regulation of various cancers, including human lung cancer.

Among them, miR-26a has been identified as a tumor-related regulator in several cancers, but its pathophysiologic properties and correlation with the development of human lung cancer remain unclear. In this study, it was determined that miR-26a expression is clearly down-regulated in human lung cancer tissues relative to normal tissues.

Meanwhile, the overexpression of miR-26a in the A549 human lung cancer cell line dramatically inhibited cell proliferation, blocked G1/S phase transition, induced apoptosis, and inhibited cell metastasis and invasion in vitro. In contrast, a miR-26a inhibitor was used to transfect A549 cells, and the inhibition of endogenous miR-26a promoted cell metastasis and invasion. In addition, miR-26a expression inhibited the expression of enhancer of zeste homolog 2 (EZH2) and transactivated downstream target genes, including disabled homolog 2 (Drosophila) interacting protein gene (DAB2IP) and human Runt-related transcription factor 3 (RUNX3),

[16] http://www.ncbi.nlm.nih.gov/gene/4086

[17] http://www.ncbi.nlm.nih.gov/pubmed/22469510

which suggests that EZH2 is a potential target of miR-26a as previously reported. In conclusion, miR-26a plays an important role as an anti-oncogene in the molecular mechanism of human lung cancer and could potentially be used for the treatment of lung cancer.

Huse et al have examined miRNA26a in gliomas. Specifically they state:

Activated oncogenic signaling is central to the development of nearly all forms of cancer, including the most common class of primary brain tumor, glioma. Research over the last two decades has revealed the particular importance of the Akt pathway, and its molecular antagonist PTEN (phosphatase and tensin homolog), in the process of gliomagenesis.

Recent studies have also demonstrated that microRNAs (miRNAs) may be responsible for the modulation of cancer-implicated genes in tumors. Here we report the identification miR-26a as a direct regulator of PTEN expression. We also show that miR-26a is frequently amplified at the DNA level in human glioma, most often in association with monoallelic PTEN loss.

Finally, we demonstrate that miR-26a-mediated PTEN repression in a murine glioma model both enhances de novo tumor formation and precludes loss of heterozygosity and the PTEN locus. Our results document a new epigenetic mechanism for PTEN regulation in glioma and further highlight dysregulation of Akt signaling as crucial to the development of these tumors.

Chario et al continue[18]:

The IκB kinase (IKK) complex is involved in transcriptional activation by phosphorylating the inhibitory molecule IκBα, a modification that triggers its subsequent degradation, enabling activation of nuclear factor kappa B (NF-κB). Importantly, recent reports indicate that multiple cytoplasmic and nuclear proteins distinct from the NF-κB and IκB proteins are phosphorylated by the catalytic subunits of the IKK complex, IKKα or IKKβ. Here, I describe how IKK subunits can have crucial roles in allergy, inflammation and immunity by targeting proteins such as SNAP23 and IRF7, but also in cancer by phosphorylating key molecules such as p53, TSC1 and FOXO3a through NF-κB-independent pathways.

Thus, these recent findings considerably widen the biological roles of these kinases and suggest that a full understanding of the biological roles of IKKα and IKKβ requires an exhaustive characterization of their substrates. The IKKα- and IKKβ-dependent NF-κB-activating pathways. On the left is the TNFα-dependent signalling pathway. Upon binding of TNFα to the TNFR1, SODD is released from the receptor and triggers the sequential recruitment of the adaptors TRADD, RIP and TRAF2 to the membrane. Then, TRAF2 mediates the recruitment of the IKK complex, composed of IKKα, IKKβ and NEMO, to the TNFR1 signalling complex. The scaffold proteins TAB2 and TAB3 subsequently bind to Lys63-polyubiquitylated substrates, such as RIP1, resulting in TAK1 and then IKKβ activations .

Whereas the receptor-proximal events that include the recruitment and activation of TAK1 rely on Lys63-linked polyubiquitylations, linear ubiquitin (Ub) chains are preferentially sensed by the

[18] http://www.sciencedirect.com/science/article/pii/S0962892409001342

UBAN motif of NEMO (not illustrated, see text for details). Hsp90 and Cdc37 are also part of the IKK complex and are required for the TNFα-induced IKK activation and shuttling of the IKK complex from the cytoplasm to the membrane. Activation of IKKβ leads to IκBα phosphorylation on specific residues, polyubiquitylation through binding of ubiquitin proteins and its degradation through the proteasome pathway.

Then, the heretodimer p50–p65 binds to specific κB sites and activates a variety of NF-κB target genes coding for pro-inflammatory cytokines (IL-6) and chemokines. A variety of proteins including TAK1, IKKβ, NEMO and p65 are also phosphorylated upon TNFα stimulation.

…NF-κB-activating pathway. Binding of CD154 triggers the classical NEMO-dependent pathway (not illustrated) and the NEMO-independent cascade. This pathway relies on the recruitment of TRAF6 and the heterodimer TRAF2–TRAF3 to the CD40 receptor. NIK is subsequently activated by phosphorylation and polyubiquitylated in a degradative manner by the E3 ligase TRAF3. IKKα homodimers are activated by NIK and phosphorylate the inhibitory molecule p100, the partial processing of which generates the NF-κB protein p52. This latter transcription factor moves into the nucleus as heterodimer with RelB to regulate the expression of genes involved in lymphoid orgagenesis or coding for chemokines (BLC) or cytokines (BAFF).

The IKKα- and NF-κB-independent pathways. Activation of the TLR9-dependent pathway triggers IKKα-mediated IRF7 phosphorylation in the cytoplasm and ultimately leads to IFN-α production. Nuclear IKKα phosphorylates the co-repressor SMRT, which triggers its nuclear export with HDAC3 and its degradation.

As a result, co-activators such as CBP are recruited and the expression of NF-κB-dependent genes is induced. CBP is also phosphorylated by IKKα in the nucleus and this modification enhances its binding to NF-κB proteins and limits the expression of the p53-dependent target genes. Upon estrogen treatment (not represented), the co-activator SRC-3 is also phosphorylated by IKKα, a modification that is required for the expression of hormone-responsive genes (cyclin D1, c-myc) and, consequently, for breast cancer cell proliferation….

The IKKβ- and NF-κB-independent pathways and their relevance in allergy and in immunity. LPS stimulation via the TLR4 in macrophages triggers the sequential activation of IRAK4, IRAK1 and TAK1 (not represented) followed by the IKKβ-mediated phosphorylation of the inhibitory molecule p105.

This latter protein is subsequently processed into p50 and releases Tpl2, the activation of which triggers the MEK-dependent cascade that will ultimately drives COX-2 gene expression via Erk1/2. In mast cells, IgE stimulation through the FcεRI leads to SNAP-23 phosphorylation which triggers degranulation and anaphylactic reactions.

The IKKβ- and NF-κB-independent pathways and their relevance in cancer. TRAF2 is targeted by the deubiquitine ligase CYLD upon stimulation by TNFα and this post-translational modification facilitates the non-degradative (Lys63-linked) polyubiquitylation of TRAF2. IKKβ also phosphorylates 14-3-3β upon TNFα stimulation and releases the 14-3-3β–TPP complex from ARE sequences found in multiple mRNAs.

As a result, those mRNAs coding for cytokines and chemokines are stabilized. TNFα also triggers the IKKβ-mediated phosphorylation of TSC1, which results in mTOR activation, S6K1 and 4EBP1 phosphorylations, and ultimately to the stimulation of angiogenesis through the transcriptional induction of the VEGF-encoding gene. FOXO3a is also a target of IKKβ and this phosphorylation triggers its nuclear export and its degradative polyubiquitylation in breast cancer cells harbouring constitutive IKKβ activity.

Dok1 is also phosphorylated by IKKβ upon TNFα stimulation and this modification positively regulates cell motility. IKKβ-mediated Aurora A phosphorylation causes its subsequent degradative polyubiquitylation, a pathway that is required for genome integrity.

The tumour-suppressor protein p53 is inactivated through an IKKβ-mediated phosphorylation and subsequent degradation through the proteasome pathway.

Zhu states:

MicroRNAs (miRNAs) belong to a class of endogenously expressed, small non-coding RNAs that cause translational repression and/or mRNA destabilization by binding to the 30-untranslated regions (30-UTRs) of the target mRNAs (1). Approximately 40% of all miRNAs are located within intronic regions of protein-coding transcriptional units (TUs) (2).

Analysis of 175 human miRNAs across 24 different human organs reveals that the expression of intronic miRNAs largely coincides with the transcription of their host TUs (3), indicating that the intronic miRNAs and their host genes may be co-regulated and are generated from a common precursor transcript. Emerging evidence suggests that intronic miRNAs may be functionally associated with their host genes. Few reports suggest that there is an antagonizing effect of the intronic miRNA on the function of its host gene, as with miR-218 (4) and miR-10 (5,6).

The secreted Slit ligands and their Robo receptors constitute a Slit–Robo signaling pathway that controls the directed migration of neurons and vascular endothelial cells during embryonic development. miR-218 is localized in the intron of the Slit gene, and similar expression patterns are observed between miR-218 and Slit in different tissues.

We conducted serum starvationstimulation assays in primary fibroblasts and two-thirds partial-hepatectomies in mice, which revealed that miR-26a/b and CTDSP1/2/L were expressed concomitantly during the cell cycle process. Specifically, they were increased in quiescent cells and decreased during cell proliferation. Furthermore, both miR-26 and CTDSP family members were frequently downregulated in hepatocellular carcinoma (HCC) tissues. Gain- and loss-of-function studies showed that miR-26a/b and CTDSP1/2/L synergistically decreased the phosphorylated form of pRb (ppRb), and blocked G1/S-phase progression.

Further investigation disclosed that miR-26a/b directly suppressed the expression of CDK6 and cyclin E1, which resulted in reduced phosphorylation of pRb. Moreover, c-Myc, which is often upregulated in cancer cells, diminished the expression of both miR-26 and CTDSP family members, enhanced the ppRb level and promoted the G1/S-phase transition. Our findings highlight the functional association of miR-26a/b and their host genes and provide new insight into the regulatory network of the G1/S-phase transition.

Segura et al (2012) state:

Melanoma incidence and associated mortality continue to increase worldwide. The lack of treatments with durable responses for stage IV melanoma may be due, at least in part, to an incomplete understanding of the molecular mechanisms that regulate tumor initiation and/or progression to metastasis. Recent evidence supports miRNA dysregulation in melanoma impacting several well-known pathways such as the PI3K/AKT or RAS/MAPK pathways, but also underexplored cellular processes like protein glycosylation and immune modulation.

There is also increasing evidence that miRNA can improve patient prognostic classification over the classical staging system and provide new therapeutic opportunities. The integration of this recently acquired knowledge with known molecular alterations in protein coding genes characteristic of these tumors (i.e., BRAF and NRAS mutations, CDKN2A inactivation) is critical for a complete understanding of melanoma pathogenesis.

Here, we compile the evidence of the functional roles of miRNAs in melanomagenesis and progression, and of their clinical utility as biomarkers, prognostic tools and potential therapeutic targets. Characterization of miRNA alterations in melanoma may provide new angles for therapeutic intervention, help to decipher mechanisms of drug resistance, and improve patient classification for disease surveillance and clinical benefit.

Zehavi et al state[19]:

We show that the expression of miRNAs from a large cluster on human chromosome 14q32 is significantly down-regulated in melanoma cell lines, benign nevi and melanoma samples relative to normal melanocytes. This miRNA cluster resides within a parentally imprinted chromosomal region known to be important in development and differentiation. In some melanoma cell lines, a chromosomal deletion or loss-of-heterozygosity was observed in the cis-acting regulatory region of this cluster.

In several cell lines we were able to re-express two maternallyinduced genes and several miRNAs from the cluster with a combination of de-methylating agents and histone deacetylase inhibitors, suggesting that epigenetic modifications take part in their silencing.

Stable over-expression of mir-376a and mir-376c, two miRNAs from this cluster that could be re-expressed following epigenetic manipulation, led to modest growth retardation and to a significant decrease in migration in-vitro. Bioinformatic analysis predicted that both miRNAs could potentially target the 3'UTR of IGF1R.

Indeed, stable expression of mir-376a and mir-376c in melanoma cells led to a decrease in IGF1R mRNA and protein, and a luciferase reporter assay indicated that the 3'UTR of IGF1R is a target of both mir-376a and mir-376c. Our work is the first to show that the large miRNA cluster

[19] http://www.molecular-cancer.com/content/11/1/44

Taveira da Cruz and Jasiulionis state:

miRNAs are non-coding RNAs that bind to mRNA targets and disturb their stability and/or translation, thus acting in gene posttranscriptional regulation. It is predicted that over 30% of mRNAs are regulated by miRNAs. Therefore these molecules are considered essential in the processing of many biological responses, such as cell proliferation, apoptosis, and stress responsiveness.

As miRNAs participate of virtually all cellular pathways, their deregulation is critical to cancer development. Consequently, loss or gain of miRNAs function may contribute to tumor progression.

Little is known about the regulation of miRNAs and understanding the events that lead to changes in their expression may provide new perspectives for cancer treatment. Among distinct types of cancer, melanoma has special implications. It is characterized as a complex disease, originated from a malignant transformation of melanocytes.

Despite being rare, its metastatic form is usually incurable, which makes melanoma the major death cause of all skin cancers. Some molecular pathways are frequently disrupted in melanoma, and miRNAs probably have a decisive role on these alterations.

Therefore, this review aims to discuss new findings about miRNAs in melanoma fields, underlying epigenetic processes, and also to argue possibilities of using miRNAs in melanoma diagnosis and therapy.

7.1.3 MiRNA Melanoma Targets

Profiling of common nevi CN and atypical nevi AN by miRNA has been reported by Holst et al. They conclude:

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression through base pairing with mRNA and which are crucially involved in carcinogenesis (the so-called oncomiRs). We compared the miRNA signature between acquired melanocytic nevi showing clinical atypia (atypic nevi, AN) and common acquired nevi (common nevi, CN).

We obtained miRNA profiles from 41 biopsies (22 AN and 19 CN) and showed that AN could be differentiated from CN on the basis of the expression of 36 miRNAs (false discovery rate <0.05). OncomiRs were present in this group, and we further confirmed the differential expression of miR-125b and let-7c by qRT-PCR. Our data suggest that miRNAs are functionally involved in the pathogenesis of nevi and possibly malignant melanoma. …

Our study showed that AN can be differentiated from CN on the basis of the expression of 36 miRNAs. Furthermore, AN showed a tendency to further clustering into two groups, which could define different subtypes of AN. The fact that AN are molecularly heterogeneous has also been

suggested by Scantolini et al. (24) on the basis of mRNA expression profiles in melanocytic lesions.

Among the differentially expressed miRNA, we found the known oncomiRs let-7a, let-7c and miR-125b. The expression pattern showing a reduced miR-125b expression indicates a more 'malignant' miRNA signature of AN in comparison with CN. Because N-RAS and P53, known to be involved in MM, can be targeted by these miRNAs (the list of other predicted targets is shown in the supplementary material), further studies should identify their functional significance in the pathogenesis of melanocytic lesions.

In another paper by Segura et al the authors find:

MiRNA signatures have potential as clinically relevant biomarkers of prognosis in metastatic melanoma. Our data suggest that molecularly based models of risk assessment can improve the standard staging criteria and support the incorporation of miRNAs into such models.

They state:

MiRNAs significantly associated with post-recurrence survival. Eighteen miRNAs found overexpressed in metastatic tissues of patients with longer survival (≥1.5 y) compared with patients with shorter survival (<1.5 y). Fold change, FDR, Cox regression coefficient (d), chromosome location, and host genes (when pertinent) are indicated.

miRNA	Score (d) Cox Regression Coefficient	Location	Host Gene
has-miR-150	−3.98	19q 13.33	Intergenic
has-miR-455-3p	−3.52	9q32	COL27A1
has-miR-145	−3.06	5q 33.1	Intergenic
has-miR-342-3p	−2.9	14q 32.2	EVL
has-miR-497	−2.87	17p 13.1	AC027763.1
has-miR-155	−2.77	21q 21.3	Intergenic
has-miR-342-5p	−2.66	14q 32.2	EVL
has-miR-143	−2.61	5q 33.1	Intergenic
has-miR-193a-3p	−2.43	17q 11.2	Intergenic
has-miR-146b-5p	−2.38	10q 24.32	Intergenic
has-miR-28-3p	−2.32	3q 28	LPP-201
has-miR-10b	−2.14	2q 31.1	HOXD3
has-miR-193b	−2.08	16 p3.12	Intergenic
has-miR-28-5p	−1.87	3q 28	LPP-201
has-miR-142-5p	−1.86	17q 22	Intergenic
has-miR-143	−1.73	5q 33.1	Intergenic
has-miR-126	−1.73	9q34.3	EGFL7
has-miR-214	−1.72	1q 24.3	DNM3

They conclude with:

Many of the miRNAs from our signature are located in genomic regions previously reported as altered in melanoma, such as loss of 9q32 (miR-455-3p) in melanoma cell lines, gain of the 5q locus (miR-145) in acral melanoma, and gain of 21q (miR-155) in uveal melanoma (Supplementary Table S8).

In conclusion, our results show the potential of miRNAs as clinically useful markers of prognosis in metastatic melanoma patients. A six-miRNA signature was able to improve risk stratification for stage III patients, suggesting that miRNAs may serve as a useful molecular adjunct to the current morphologic staging system in identifying high-risk patients who might benefit from adjuvant therapy.

Differential expression of most miRNAs from the predictor signature was also observed in the matched-pair primary tissue, suggesting that the miRNA signature may also play a role in prognosis of early lesions. Further studies with a larger cohort of primary melanoma patients are needed to better define the role of the signature in predicting the development of aggressive disease.

Recent work by Luo also details similar markers (p 66).Luo also details a more comple causative analaysis of miR-101. Luo also does a detailed analysis of MITF as well as EZH2 which we have discussed in previous literature analyses. Luo concludes:

To test whether miR-101 is able to down-regulate MITF protein expression, we transfected Ma-Mel-79b and -86b cells with miR-101 mimic and performed Western blot analysis using antibody against MITF. As shown in ***Figure 14A****, MITF protein was decreased to 62 % and 56 % in Ma-Mel-79b and -86b cells at 72 hr after transfection of miR-101.*

We also tested the expression of EZH2, which has been reported to be an oncogene and a target of miR-101 in various types of cancer (87-89, 92, 93). Indeed, overexpression of miR-101 down-regulated EZH2 in the two tested melanoma cell lines. Interestingly, overexpression of miR-101 in both cell lines also caused a cleavage of poly-(ADP-ribose) polymerase (PARP) which is one of the targets of caspase-3 and serves as an indicator for apoptosis

7.1.4 Therapeutic Application

Kota et al present an analysis of the application of miRNA therapeutics. They state:

Therapeutic strategies based on modulation of microRNA (miRNA) activity hold great promise due to the ability of these small RNAs to potently influence cellular behavior. In this study, we investigated the efficacy of a miRNA replacement therapy for liver cancer. We demonstrate that hepatocellular carcinoma (HCC) cells exhibit reduced expression of miR-26a, a miRNA that is normally expressed at high levels in diverse tissues.

Expression of this miRNA in liver cancer cells in vitro induces cell-cycle arrest associated with direct targeting of cyclins D2 and E2. Systemic administration of this miRNA in a mouse model of HCC using adeno-associated virus (AAV) results in inhibition of cancer cell proliferation, induction of tumor-specific apoptosis, and dramatic protection from disease progression without toxicity. These findings suggest that delivery of miRNAs that are highly expressed and therefore

tolerated in normal tissues but lost in disease cells may provide a general strategy for miRNA replacement therapies.

7.2 EXOSOMAL TARGETS

As Pfeffer et al have noted:

A number of differentially regulated miRNAs identified by NanoString human V2 miRNA array were validated by quantitative PCR. Significantly, miR-17, miR-19a, miR-21, miR-126, and miR-149 were expressed at higher levels in patients with metastatic sporadic melanoma as compared with familial melanoma patients or unaffected control subjects. Surprisingly, no substantial differences in miRNA expression were detected between familial melanoma patients (all inclusive) and unaffected control subjects.

The miRNAs differentially expressed in the different patient cohorts, especially in patients with metastatic melanoma, may play important roles in tumor progression and metastasis, and may be used as predictive biomarkers to monitor remission as well as relapse following therapeutic intervention…we investigated miRNA signatures of plasma-derived exosomes from familial and sporadic melanoma patients and unaffected family members. Several miRNAs were differentially expressed in plasma-derived exosomes, which may form the basis for future studies on their applicability as predisposition biomarkers and potential chemoprevention targets.

An important aspect of our studies was the finding that miR-17, miR-19a, miR-21, miR-126 and miR-149 were expressed at higher levels in plasma-derived exosomes from patients with metastatic melanoma.

Many of these miRNAs have been associated with various cancers, and in some cases with melanoma specifically.

For example, using a high-throughput approach, miR-17 was identified as a potential oncogenic miRNA in melanoma. Previous studies demonstrated that miR-17 is highly expressed in leukemia and lung cancer, and it promotes cell proliferation by targeting p21 as well as PTEN and RB. Also, increased expression of miR-19a leads to increased melanoma invasiveness. MiR-19a is an important member of the oncogenic miR-17-92 cluster.

MiR-19a is upregulated in acute myeloid leukemia, colorectal cancer and gastric cancer, and is believed to act through promoting tumor growth and metastasis.

MiR-21 is frequently upregulated in human tumor cells where it appears to play an important role in the oncogenic process through its association with increased proliferation, low apoptosis, high invasion and metastatic potential. However, miR-21 is also upregulated in the inflammatory response, which also may play an important role in tumor progression as well as in tumor elimination. We recently found that IFN upregulated miR-21 expression in both melanoma and prostate cancer cells, which diminished their apoptotic sensitivity. In contrast, knockdown (KD) of miR-21 expression enhanced apoptotic sensitivity to IFN as well as to several chemotherapeutic agents.

Consistent with these findings, miR-21 inhibition in human melanoma cells increases expression of the PTEN target gene, leading to suppression of AKT phosphorylation and subsequently increased Bax/Bcl-2 ratio. Most interestingly, using mouse B16 melanoma, we found that while the parent cell line exclusively formed large tumors in the lungs of tail-vein injected mice, miR-21 KD cells formed only small lung tumors, and mice injected with miR-21 KD cells exhibited markedly prolonged animal survival. Elevated miR-126 expression has been observed in normal melanocytes and primary melanoma cell lines, while it was reportedly reduced in metastatic melanoma. Overexpression of miR-126 was found to enhance melanogenesis.

MiR-149 is upregulated in melanoma cells and is expressed in response to p53 activation. However, miR-149 provides a mechanism to bypass the induction of apoptosis by p53 activation by directly targeting glycogen synthetase-3α and thereby stabilizing MCL-1…

7.3 METASTASIS AND MIRNA

Metastasis is a complex process which we have examined before[20]. As Gajos-Michniewicz and Czyz have noted:

Normal melanocytes produce melanin, which is transferred in melanosomes to keratinocytes to induce skin pigmentation. Melanoma cells retain the ability to transfer melanosomes to neighboring cells, which contributes to the formation of the tumour niche within the dermis. Dror et al. have shown that melanoma cells communicate with fibroblasts via melanosomes that are released into the dermis prior to melanoma cell invasion.

*Moreover, they have demonstrated melanosomal transport of **miR-211**, which contributes to the reprogramming of primary fibroblasts into cancer-associated fibroblasts (CAFs). CAFs play an important role in the progression from primary to metastatic melanoma. Increased proliferation of CAFs has been shown to be associated with a reduced level of IGF2R (insulin growth factor 2 receptor), and IGF2R mRNA was found to be a direct target of miR-211.*

Moreover, miR-211 transfected into fibroblasts upregulates phospho-ERK, which indicates activation of the MAPK signaling pathway. The spectrum of miRNAs transferred by melanoma-derived melanosomes is different from melanosomes secreted by melanocytes, and lower miR-211 levels have been observed in the dermis of normal skin compared with melanoma in situ. These findings suggest that melanoma cells are capable of modulating the stromal niche during the early phase of the disease by altering the phenotype of dermal fibroblasts. Thus, blocking the release of melanoma-derived melanosomes might forestall early dermal changes.

*Another mutation-independent mechanism that underlies the initiation of melanoma metastasis during the transition from radial to vertical growth involves **miR-222/221**, NOTCH, and MITF. In melanoma cells that are present in a NOTCH ligand-free microenvironment, the transcription factor MITF represses the miR-222/221 promoter. During radial growth, which occurs when melanoma cells establish contact with distal differentiated keratinocytes that express NOTCH*

[20] https://www.researchgate.net/publication/330521548_NSD2_PCa_and_Metastasis

ligands, the activated intracellular domain of NOTCH interferes with MITF binding to the miR-222/221 promoter.

The increased expression of miR-222/221 therefore enables the acquisition of invasion capabilities. It has been shown that the level of oncomiR-222/221 inversely correlates with the level of tumour suppressor miR-126/miR-126 during melanoma progression. Felli et al. have reported high expression of miR-126/126 in melanocytes and primary vertical growth phase melanomas, with its decrease in subcutaneous and lymph-node metastases, whereas miR-221/222 expression has been almost undetectable in melanocytes and gradually increased during melanoma progression. Protein analyses have revealed the reverse expression pattern of several factors, targeted by miR-126/126 and induced by miR-222/221, or the opposite. Interestingly, the dual regulation of AP2α (activating protein 2 alpha), a transcription factor that is lost during transition from primary to local and metastatic dissemination, has been revealed.

While AP2α plays a role of a transcriptional activator of tumour suppressor miR-126/126, AP2α transcripts are targeted by oncogenic miR-222/221. Alterations in this auto-sustaining loop during melanoma development may contribute to changes in the expression of several pro-oncogenic factors, including cell adhesion molecules, extracellular matrix (ECM) degrading enzymes, angiogenic and survival proteins. Increased expression of miR-221/222 in melanoma cell lines has been associated with induced proliferation, migration, and invasion of melanoma cells in vitro and in vivo compared to normal melanocytes. The expression of miRNA-221/222 is transcriptionally downregulated by PLZF (promyelocytic leukaemia zinc finger) or antagomir-221/222 treatment.

miR-222 is part of the melanoma exosomal cargo, and exosomes released by miR-222-overexpressing cells are taken up by recipient primary melanoma cells to promote tumour growth through the activation of the PI3K/AKT pathway, and downregulation of the tumour suppressor p27. Transcripts of c-KIT and the cyclin-dependent kinase inhibitor p27 are targets of miR-221/222. miR-221-induced attenuation of p27 and c-KIT has been shown in another study . Inactivating point mutations in p27 are rare, and p27 is mostly regulated at the posttranscriptional and posttranslational levels. Thus, a proposed miR-221/222-based mechanism that blocks p27 translation represents an additional oncogenic program that triggers an abnormal cell cycle rate in melanoma cells.

8 MEASUREMENT TECHNIQUES

miRNAs are present in cells as well as bodily fluids. We examine the methods available to detect them and subsequently to measure their intensity, or density relative to some established metric. If we would anticipate using them as markers for diagnosis or prognosis, then we would also anticipate that we can "measure" them in some manner. It is not just the presence of some miRNA, which all too often is the case, but the intensity of that presentation. Thus understanding how these numbers are obtained, and more importantly what the problems resulting in their determination may be, that allows us to develop robust metrics.

Let us begin with a simple example. Suppose we have a PSA measurement, a PSA velocity and a % Free PSA. We are then asked to obtain a probability that the patient has PCa. Thus we must map these three numbers into a probability. Note this is for a fixed organ and the inputs are related solely to that organ so there would be no ambiguity. There are such tests and they are used.

Now let us assume we have measurements on N miRNAs, and that these measurements are relative from some PCR type test, which we shall explain. Namely we have the measurement set:

$$\{x_1.....x_N\}$$

Then we must find what this maps into. It could map into any one of k organs and for each organ it gives a probability of a disease state. In essence, what we seek is how to measure the x values and how many of them and then how to calculate the probabilities and how many of them. There are many ways to accomplish this but in general it is a classification problem with a probabilistic output.

8.1 MEASUREMENTS OF miRNA

There are many ways in which we can measure miRNAs. The technology has developed greatly in the past decade. An excellent paper by Pritchard et al presents an overview.

First Pritchard et al comment on recovery of miRNA. This is an important observation in that it may generally be readily obtained:

It is possible to extract high-quality miRNA from a wide range of cell and tissue sources, including cell lines, fresh tissues, formalin-fixed paraffin-embedded (FFPE) tissues, plasma, serum, urine and other body fluids.

The principles for isolating miRNA are, in general, the same as for isolation of total RNA, except that miRNA isolation protocols are often slightly modified to retain (and sometimes to enrich) the small RNA fraction[28]. Widely used commercially available products are generally based on chemical extraction using concentrated chaotropic salts, such as guanidinium thiocyanate (for example, Trizol and QIAzol reagents), followed by a solid-phase extraction procedure on silica

columns. miRNAs may also be analysed using total RNA isolated by traditional chemical extraction with Trizol

Pritchard et al summarize the many profiling techniques. Specifically on Figure 3 of their paper they depict the following:

Approaches to microRNA profiling.

a. Quantitative reverse transcription PCR (qRT-PCR). In TaqMan qRT-PCR, the reverse transcription reactions use stem–loop primers that are specific to the 3' end of the microRNA (miRNA) for specificity (top left). Amplicons are generated using an miRNA-specific forward primer and a reverse primer. As the DNA polymerase proceeds along the template, the TaqMan probe is hydrolysed and fluorescent dye is freed from the quencher, resulting in light emission (top middle). In SYBR-green-based qRT-PCR, miRNA is typically polyadenylated at the 3' end, and oligo-d(T) is used as a reverse transcription primer (bottom left). An miRNA-specific forward primer and a reverse primer that anneals to the 3' portion of the miRNA sequence as well as to the poly(A) tail enable PCR amplification with dsDNA-intercalating SYBR green dye as the detector (bottom middle). Both TaqMan and SYBR-green-based qRT-PCR are available in 'array' format (right).

b. miRNA microarray. DNA-based capture probes (which may or may not incorporate LNA-modified bases) are used to capture fluorescently tagged miRNAs; this is followed by scanning of slides and quantification of fluorescence.

c. Nanostring nCounter. A bridge oligonucleotide templates ligation of an miRNA to a specific tag. Capture and detection is done by two target-specific probes: a 3! capture probe containing biotin to allow adsorbance to the solid phase via streptavidin and a second 5! reporter probe with an individual colour-coded sequence. No amplification or labelling of miRNA is required with this method.

d. RNA sequencing (RNA-seq). Currently established RNA-seq platforms begin with reverse transcription of miRNA to a cDNA library. Adaptor ligation then allows the library either to be affixed to a solid phase, as in the Illumina platform, or to beads for emulsion PCR, as in the Roche and ABI platforms (for details of sequencing chemistry

8.2 SOURCES

Let us first examine the sources of miRNA for which we seek to examine. From Shigeyasu et al:

The first interpretation of the term "liquid biopsy" in cancer originated in 2010 when circulating tumor cells (CTC) were proposed as alternatives to conventional breast cancer biopsy for prognosis and evaluation of treatment responses (7). Subsequently, clinical applications of liquid biopsies have diversified from detecting early-stage cancer to monitoring tumor progression, assessing tumor heterogeneity and residual disease, and potentially monitoring therapeutic response to various surgical and chemotherapeutic interventions. …

These noninvasive but technologically sophisticated applications can be incorporated into existing treatment practices to decrease gastrointestinal cancer– associated mortality. Recently, the sources of liquid biopsies have expanded beyond blood to include other body fluids including stool, urine, and saliva, which may directly detect cancer in associated organs.

Likewise, the term "biopsy" has broadened to encompass other subcellular components including circulating tumor DNA, ncRNAs, predominantly miRNAs, proteins, and extracellular vesicles that can be used as targets for evaluation in gastrointestinal cancer. In this regard, despite the initial enthusiasm for the identification of CTCs and ctDNA in liquid biopsies from patients with cancer, accumulating data indicate that although these targets offer a high degree of cancer specificity, both entities are scarce in circulating biofluids and may be inadequate as clinically applicable diagnostic biomarkers.

On average, ctDNA represents less than 1% of the total circulating free DNA found in biofluids, whereas in patients with cancer, the ratio of CTCs to white blood cells is approximately 1:1 million. Accordingly, a study that evaluated the ability of ctDNA to identify specific mutations in individuals' primary tumors reported success in only 73% of colorectal, 57% of gastroesophageal, and 48% of pancreatic cancers.

These results may be considered somewhat disappointing considering that each of these mutations was known a priori before screening. Consequently, other molecules derived from tumor cells, such as ncRNAs, are far more abundant than ctDNA or CTCs in biofluids, arerelatively stable in a variety of biological fluids, and are frequently dysregulated even in the earliest stages of cancer. These characteristics argue in their favor for further development as noninvasive liquid biopsy biomarkers for human cancers. Circulating miRNAs as cancer diagnostic biomarkers In 2008, tumor-associated miRNAs (miR-155, miR-210, and miR-21) were first discovered to be upregulated in serum of patients with lymphoma.

To date, more than hundreds of miRNAs have been identified as potential diagnostic targets in various cancers. Circulating miRNAs possess unique features making them likely candidates for development as disease-specific biomarkers. miRNAs are generally stable in blood and other body fluids due to their small size and their ability to escape from RNase-mediated degradation, and nearly 10% of miRNAs are either secreted in membranous nano-sized vesicles called "exosomes," whereas the remaining 90% are stabilized and packaged with other proteins, such as argonaute-2 (Ago2), highdensity lipoprotein (HDL), and other RNA-binding proteins. Furthermore, both exosomal- and Ago2/HDL-attached miRNAs are actively secreted from living cells, whereas the majority of ctDNA is passively released by apoptotic or necrotic cells …

A recent study demonstrated that miRNAs offer superior sensitivity and specificity compared with ctDNA for diagnosing colorectal cancers. Collectively, miRNAs appear to be promising candidates as liquid biopsy– based cancer biomarkers. Nevertheless, several obstacles must be overcome before miRNAs can be recognized and adopted as clinically relevant cancer diagnostic biomarkers. In particular, the lack of disease and organ specificity and uncertainty of normalization are among the most critical issues.

With the significant body of literature gathered on circulating miRNAs in gastrointestinal cancers and the availability of high-throughput microarray and RNA sequencing profiling from

serum and plasma samples from patients with cancer, we are very likely bound to identify robust miRNAs as potential cancer diagnostic markers in the near future. The diagnostic potential of many circulating miRNAs has been assessed in a variety of cancers…

We can look back upon the various malignancies we discussed and see a multiplicity of cancers for a specific miRNA.

8.3 ASSAY METHODS

As (miRNAs) are a comparatively small target to experimentally verify and quantify in the lab.

Although bioinformatics plays a big part in identifying putative miRNAs, they also need to be experimentally verified in the lab. A range of techniques has been developed to overcome the challenges of miRNA profiling.

Here we review the most popular methods currently in use. Use our table to see at a glance which method is most suited for your experiments and click on a technique for more detail.

	When to use	***Benefits***	***Drawbacks***
qPCR	Small scale experiments (1–2 samples or miRNAs)	Established protocols High sensitivity and specificity	Labor intensive to scale Requires quality miRNA annotation
miRNA arrays	Larger studies (has been used for up to 900 samples)	Established protocols Purpose built analysis tools	Least quantitative Requires quality miRNA annotation
RNA-seq	Discovery phase research	Whole genome analysis Single base resolution Does not require miRNA annotation	Less sensitive than qPCR Requires most input material Good degree of technical and bioinformatics skill necessary
Multiplex miRNA profiling	Multiplex studies (68 miRNAs, has previously been used for up to 600 samples)	High sensitivity and specificity Straightforward data analysis Can be used on crude biofluids Requires standard lab equipment	Not ideal for small scale experiments of 1–2 samples Requires quality miRNA annotation

8.3.1 miRNA qPCR assays

One of the most popular techniques for validating and accurately quantifying miRNAs is quantitative real time PCR (qPCR). As well as being sensitive and quantitative, qPCR is also relatively inexpensive and flexible making it the preferred choice for validating novel miRNAs and for use in relatively small experiments.

This technique begins with the conversion of miRNA to cDNA. With the length of a miRNA being comparable to that of a typical DNA primer, cDNA synthesis from miRNAs presents its own challenges. The solution to this is to make the molecule longer, either by incorporating a poly(A) tail or stem-loop structure.

Once miRNA has been converted to cDNA it can be assayed using the same approach as a conventional qPCR experiment. Amplification is initiated with an miRNA-specific primer and a stem-loop/poly(A) primer. Either SYBR® Green or a TaqMan[21] probe is used to detect the amplified product.

However, qPCR has its limitations: large experiments can become quite labor intensive to perform. Moreover, unlike conventional qPCR, only one flanking primer can be specific to the miRNA, so care must be taken to ensure only one product is being amplified, especially when using SYBR® Green.

The short template length can prove a particularly problematic issue when trying to distinguish miRNAs that may only differ by a handful of bases; melting temperatures can be very low and very similar. The use of novel probes such as locked nucleic acids have been developed that mitigate specificity issues, but sample throughput remains a limitation for large studies[22].

8.3.2 miRNA arrays

Arrays are typically chosen for larger studies covering multiple miRNA targets. While they are the least quantitative of the three miRNA assay methods, conventional DNA oligonucleotide arrays are a relatively inexpensive way to measure hundreds of targets at once.

Thousands of probes can be easily spotted on slides, or built up by photolithography, potentially enabling the parallel tracking of all known miRNAs. Arrays are probed by hybridizing fluorescently labeled DNA or RNA samples. The brightness of individual spots can be used to infer relative changes in expression between samples.

As with qPCR, distinguishing similar sequences may be problematic, but careful selection of control probes, stringent washing and analysis can mitigate the issue. The maturity of the array platform is a significant benefit here, as there are well developed protocols and purpose built analysis tools available.

8.3.3 RNA-seq

This method of miRNA quantification uses the high-throughput capability of next-generation sequencing (NGS) platforms. While it cannot quantify miRNA levels with the molar resolution of qPCR, deep sequencing of miRNA does have the advantage of being able to sample all miRNAs present in a sample, whether the researcher knows the sequence or not, making it an ideal discovery tool. Furthermore, as sequences are read directly, RNA-seq can distinguish closely related miRNAs and isoforms.

[21] https://www.thermofisher.com/us/en/home/life-science/pcr/real-time-pcr/real-time-pcr-learning-center/real-time-pcr-basics.html

[22] https://pubs.acs.org/doi/pdf/10.1021/bi0485732

This method has a number of advantages: it can distinguish miRNAs at a single base resolution, it doesn't require upfront knowledge of an miRNA's existence, it can determine relative miRNA expression levels and, better yet, tagged libraries can be used for multiplexing.

However, of all the methods discussed, NGS does require the most input material, and even then miRNA-seq can't match the sensitivity of qPCR.

Library construction, especially amplification, is potentially a sizeable source of bias and requires a good degree of technical skill to undertake *see Baker). Data analysis can also be relatively challenging, especially for a labs that may not have a resident bioinformatician.

8.3.4 Multiplex miRNA profiling

Multiplex miRNA profiling assays using Firefly particle technology are a more recent addition to the range of tools available to assay miRNAs. A key benefit of this technique is its ability to allow the validation of multiple miRNAs across a range of samples, without the labor intensive workflow or large sample requirement of other techniques.

This technique is dependent on hydrogel particles that contain custom selected probes against target miRNAs. miRNAs bind to these probes and are then ligated to adaptor sequences for detection or pre-detection amplification.

Particles are optimized for use with common bench-top flow cytometers, allowing detection without specialized lab equipment. In addition, data analysis is relatively straightforward and does not require advanced bioinformatics skills.

This technology can be used directly with crude biofluids, and preparation of an RNA library is not necessary. The high sensitivity of the assay means that miRNA profiling can be achieved from input of as little as 10 µl of plasma or serum, or 100 pg purified RNA.

Which tool is best? In truth, each of the techniques described can be considered largely complimentary; experimental aims and material considerations will likely dictate which tool a lab opts for first.

Studies focused on one or two miRNAs with relatively few test groups are likely to opt for qPCR as the primary assay, whereas experiments trying to discover new miRNA variants will look to an NGS solution. For larger studies examining multiple miRNAs at once, microarrays or multiplex miRNA assays using Firefly particle technology are more suitable.

Whatever the choice, given the difficulties involved in handling, verifying and interpreting miRNA data, the consensus view is that best practice is to verify the results of one technique by using a second where possible [23].

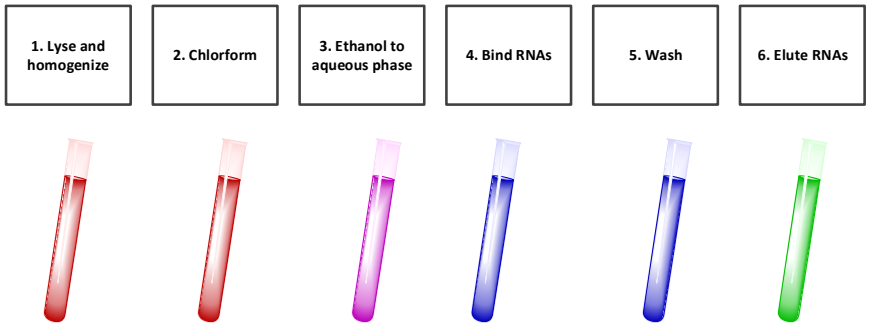
8.4 EXTRACTION

[23] Baker, MicroRNA profiling: separating signal from noise, Nature Methods volume 7, pages687–692(2010)

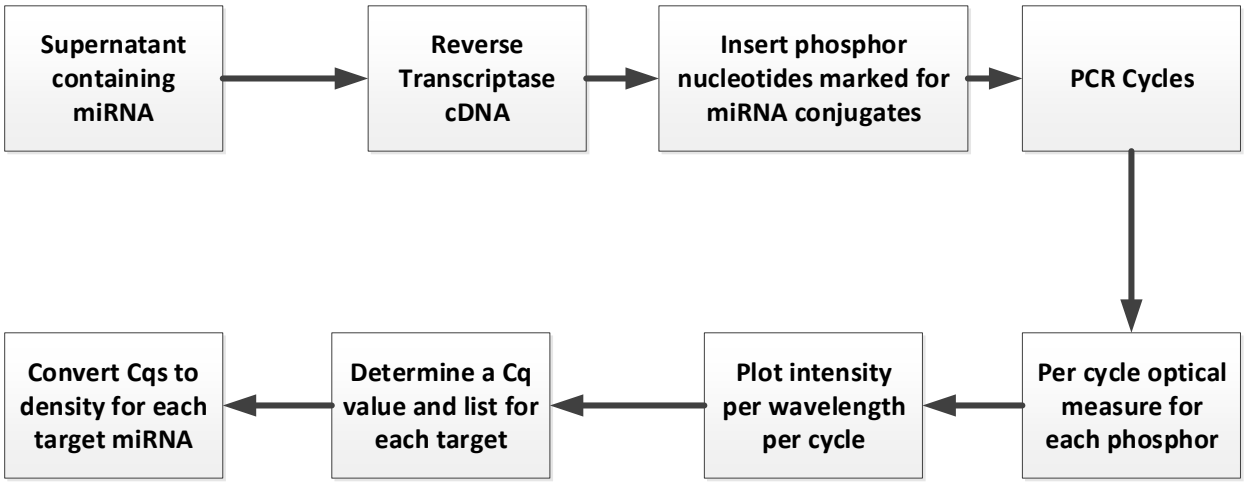
Obtaining miRNAs for determination can be a somewhat straightforward process. For miRNAs in cells we must first lyse the cell and extract the miRNA. This applies equally to exosome miRNAs which are encapsulated. However, for free miRNA as may be the case for a liquid biopsy application we have the miRNA free in the medium. miRNAs have been found to be robust and do not breakdown as is the case for free RNA or DNA. They retain their overall structure and thus can be identified rather simply.

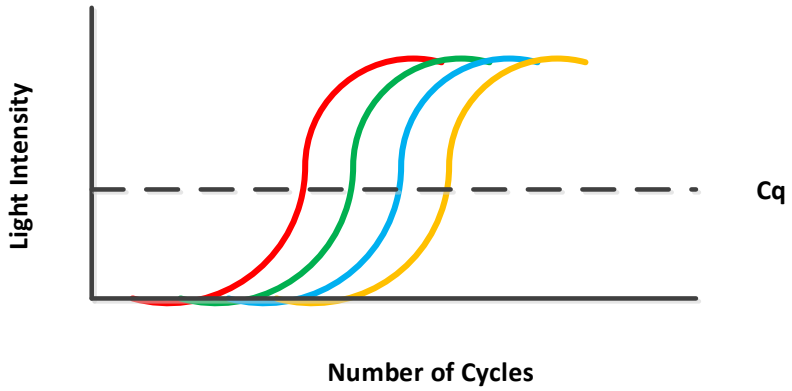
We now proceed to provide a simplified description of miRNA identification and quantification and focusing on the PCR scheme discussed briefly above. This presentation is descriptive but not fully dispositive and the reader is referred to the related literature for complete details.

The process is described by the steps below. Simply we extract the miRNA, often already extracted, the bind it, reverse transcriptase it to get cDNA and then read the DNA. This also allows a relative measurement of the intensity of the miRNA.



The following descriptive again repeats briefly what we have shown above. What we note is that depending on the number of PCR cycles, or effectively the number of cDNAs, we have markers which can measure relative intensity of light and thus in turn of the substance. From this we can using the number of cycles infer relative intensity of the separate miRNAs marked. This is shown by the graph below the process.





A key factor is the C_T value. We use this as a baseline to measure the intensity depending on PCR cycles.

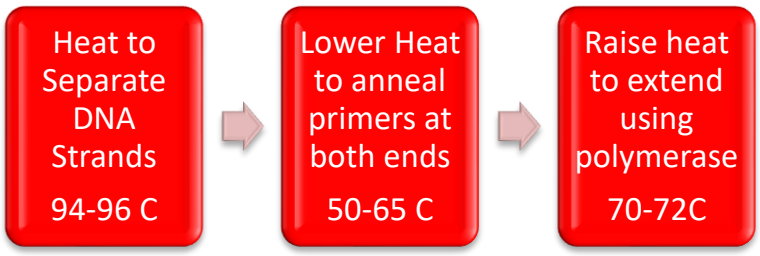
From Qiagen[24]:

CT is the abbreviation for Threshold Cycle and is defined as the calculated cycle number at which the PCR product crosses a threshold of detection. This threshold line is either automatically set by the software algorithm of the real-time PCR instrument or can be manually adjusted. The CT values are essential for quantitation since the standard curve is generated by plotting the CT values versus the logarithmic concentration. A positive unknown sample will also be assigned a specific CT value and by comparison with the standard curve a particular concentration can be calculated

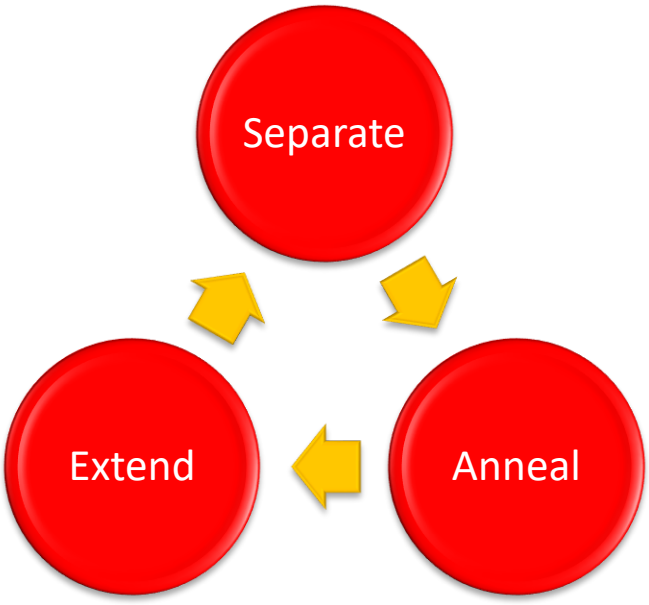
The PCR process is described below. We heat the cDNA to separate, cool and anneal primers, heat and use polymerase to double the cDNA. This is the fundamental way each cycle proceeds. Each cycle then doubles the number of cDNA. Each cDNA has a phosphorescent marker and the

[24] https://www.qiagen.com/no/resources/faq?id=c556c4f2-4c90-4cbc-839f-0a8d65d67faf&lang=en

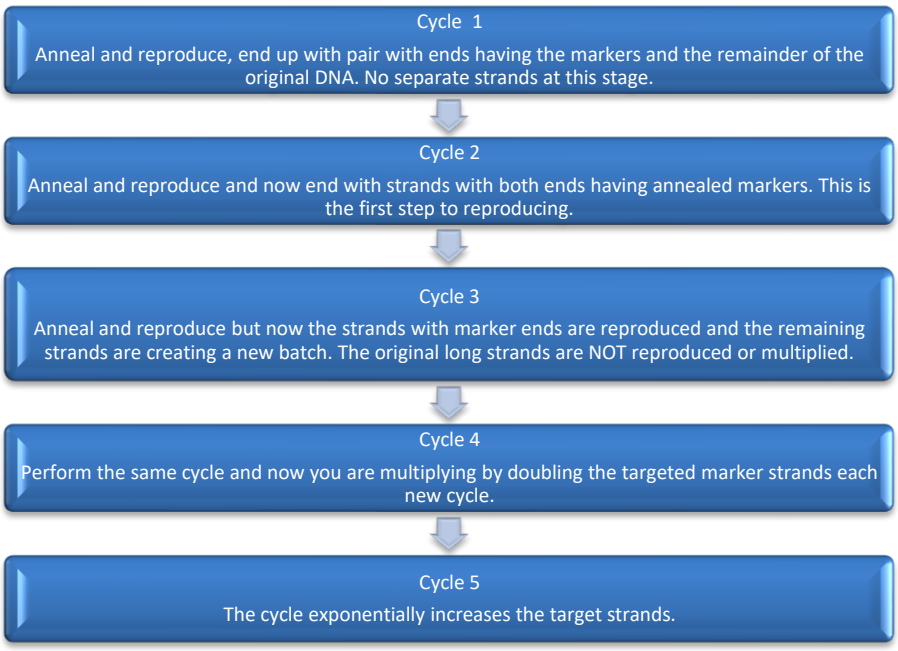
more cDNA, via cycles, the brighter the market. For low density initial cDNA we take longer to get to the desired level of intensity.



We re[eat the process in the following graphic.

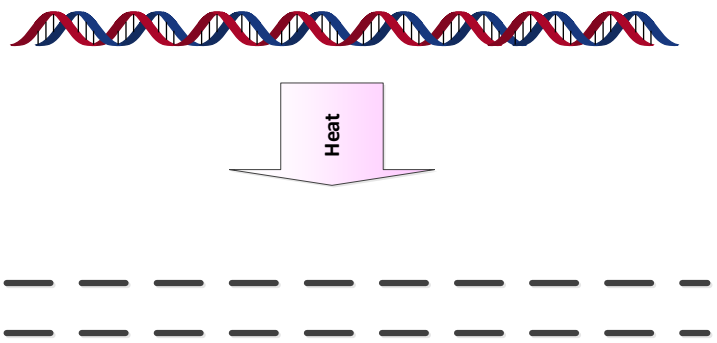


The details on PCR are laid out below:

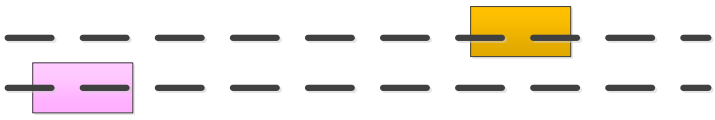


Now let us go through the steps starting with one strand of cDNA as shown below. First, heat and separate:

Step 1: Heat DNA to 94-96C to separate strands

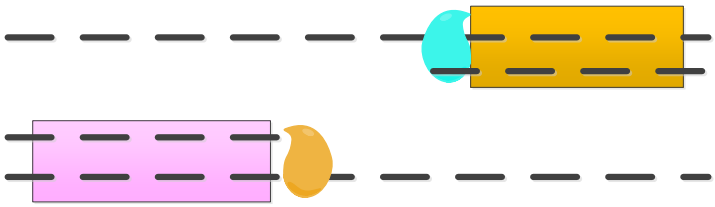


Second anneal and use polymerase to create new strands.



Step 2: Lower the heat to 50-65C to anneal primers left and right. Then raise to 70C to anneal

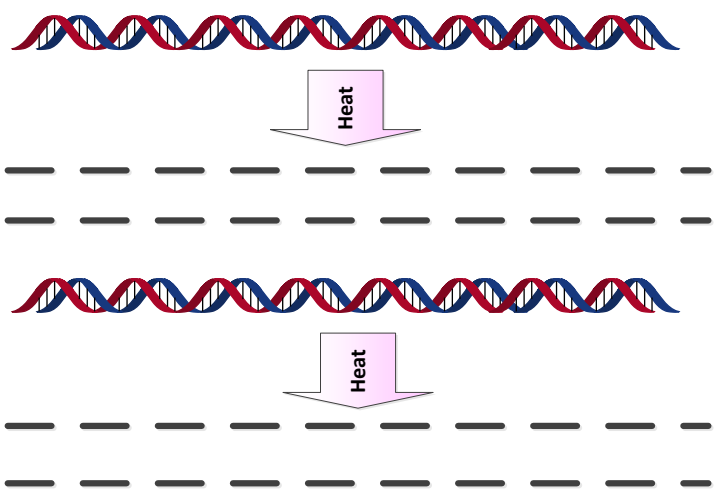
Next, with the new strands repeat the process;



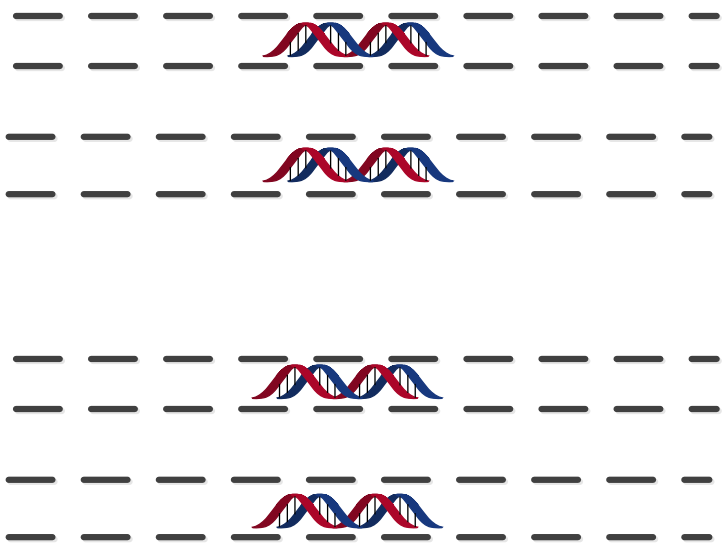
Step 3: At 72C use polymerase to extend primers across the open strands

Repeat again:

Step 4: Begin the process again doubling each time

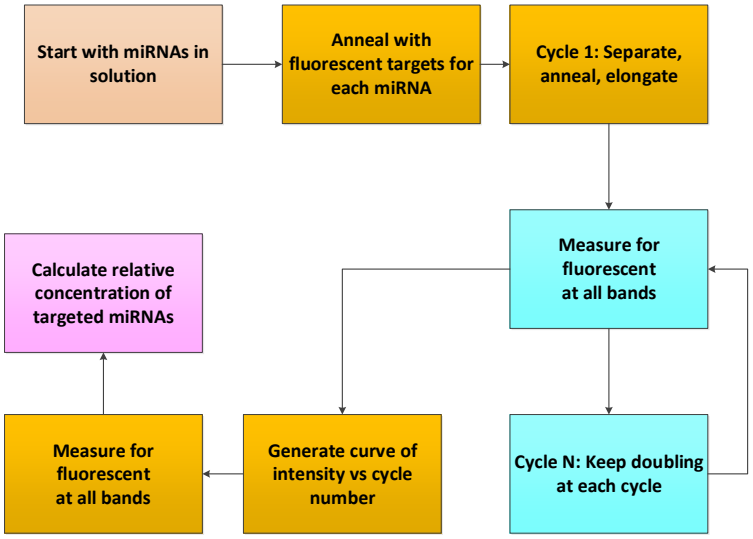


Keep repeating so as to double the cDNA each time as below:

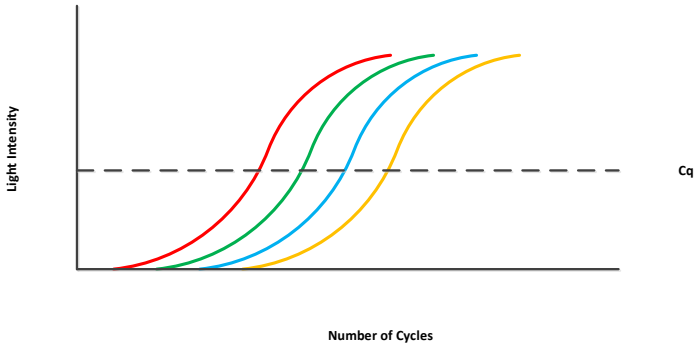


Steps 1-4: Separate, anneal, polymerase, repeat. Doubles the cDNA each time!

Finally, we reiterate the process.



From this we can obtain light density versus number of cycle plots for each target annealing as shown below:



Note: A cycle is a temperature/doubling cycle. Thus we start with 1, then 2, then 4, 8, 16 etc. Each is tagged with a fluorescent and they grow in number until they can be readily seen and measured in intensity. Each of the above curve is for a different miRNA

8.5 QUANTIFICATION

The target for RT-PCR analysis is the measurement of CT ate some defined level of reproduction in the PCR cycles. As Qureshi and Sacan have noted:

There are two main tools used to quantify the expression of miRNAs: microarrays and real-time polymerase chain reaction (RT-PCR). RT-PCR returns the number of cycles that the samples underwent before they were detected, reported as a value known as the Cycle Threshold (CT). The CT values vary logarithmically with expression levels.

There are several methods of normalizing the data and calculating the fold-change of each gene between samples. For convenience, in this presentation the terms, "miRNA" and "gene," are used interchangeably in the context of RT-PCR. ΔCT values are calculated by subtracting the CT value of the endogenous control for a given sample (or the mean of the CT values of the endogenous controls if more than one exist) from the CT value of the gene for the given sample.

In the calculation of ΔCT values we refer to the number subtracted from the raw CT values of each gene as the CT0. The ΔΔCT is calculated by subtracting the ΔCT of an experimental sample from a control sample. Fold change is calculated by raising 2 to the power of the negative ΔΔCT value, since CT values are related to the amount of miRNA or gene logarithmically. The relationship between CT, ΔCT, ΔΔCT, and Fold Change (FC) are given by the equations below.

$$CT = CT - CT_0$$

$$\Delta\Delta CT = \Delta CT - \Delta CT_{control}$$

$$FC = 2^{-\Delta\Delta CT}$$

As Ho et al have noted:

Substantial efforts have been devoted to in vitro testing of candidate chemotherapeutics by profiling transcriptional changes across the collection of NCI-60 cell-lines. A work-flow with reagents that enable the direct quantification of RNA of different molecular sizes simultaneously in the same sample without laborious total RNA isolation will invariably increase the throughput and accuracy of the study. MicroRNAs (miRNAs) are known to regulate most cellular functions, acting post transcriptionally by repressing numerous eukaryotic mRNAs. Recent findings on the remarkable stability of miRNA prompted us to investigate the feasibility of quantifying the expression levels of both mRNA and miRNA directly from cell lysates (cell-to-Ct).

Multidimensional analyses of the expressions of mRNA and miRNA across seven NCI-60 cell lines and multiple reagents were conducted to assess the performances of these reagents and workflows for cell-to-Ct measurements using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Quantification of RNA species using lysates prepared from an in-house and one of the commercial reagents demonstrated comparable performance to those prepared by the more laborious and conventional method of using guanidinium-phenol-chloroform.

Additionally, miRNA was found to be highly stable in the cell lysates when incubated at room temperature for prolonged period of time and subjected to multiple freeze-thaw cycles. In summary, this study demonstrated significant differences in pre-analytical performance of a variety of commercially available reagents and described a cost-effective reagent useful for rapid, scalable, and high throughput workflow for the detection of mRNA and miRNA from the same biological sample.

Now Bustin et al (2009 and 2010) have delineated a long set of concerns regarding repeatability of the measurement of miRNAs. They note:

Currently, a lack of consensus exists on how best to perform and interpret quantitative realtime PCR (qPCR) experiments. The problem is exacerbated by a lack of sufficient experimental detail in many publications, which impedes a reader's ability to evaluate critically the quality of the results presented or to repeat the experiments.

The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines target the reliability of results to help ensure the integrity of the scientific literature, promote consistency between laboratories, and increase experimental transparency. MIQE is a set of guidelines that describe the minimum information necessary for evaluating qPCR experiments. Included is a checklist to accompany the initial submission of a manuscript to the publisher.

By providing all relevant experimental conditions and assay characteristics, reviewers can assess the validity of the protocols used. Full disclosure of all reagents, sequences, and analysis methods is necessary to enable other investigators to reproduce results. MIQE details should be published either in abbreviated form or as an online supplement.

There clearly is a reproducibility issue.

The application to single cell analysis has been discussed by Wang et al (2019) and is a challenging but essential direction. Some of the challenges have been detailed by Pritchard et al

One major approach relies on reverse transcription of miRNA to cDNA, followed by qPCR with real-time monitoring of reaction product accumulation (known as 'realtime PCR'). An appealing aspect of this approach is the ease of incorporation into the workflow for laboratories that are familiar with real-time PCR.

In order to scale this approach for miRNA profiling, reactions are carried out in a highly parallel, high-throughput form (that is, hundreds of qRT-PCR reactions measuring different miRNAs using the same reaction conditions). Commercially available customizable plates and microfluidic cards can be designed either to examine a small set of miRNAs (for example, those that regulate a pathway of interest) or to provide more comprehensive coverage.

For large-scale miRNA profiling (that is, for hundreds of miRNAs) using qRT-PCR, several medium-throughput platforms are available that use pre-plated PCR primers that are typically distributed across multi-well dishes or alternatively across microfluidic cards containing nanolitre-scale wells (FIG. 3a). Two common strategies used for priming the reverse transcription reaction to generate cDNA are enzymatic addition of a poly(A) tail and generation of a reverse transcription primer binding site using a stem–loop primer (FIG. 3a).

A hurdle in performing highly parallel qRT-PCR is that optimal reaction conditions may vary substantially between miRNAs owing to sequence-specific differences in primer annealing. Although different vendors have sought to solve this problem using various approaches, one effective strategy has been the incorporation of locked nucleic acids (LNAs) into primers to standardize optimal miRNA primer hybridization conditions for the hundreds of PCR assays that are to be run simultaneously.

As Tiberio et al have noted:

Sequencing-based quantification of miRNAs is a relatively new technology compared to qRT-PCR and microarrays, and there is a minor standardization of the optimal workflow to be used. At present, the standard method to normalize miRNA-seq data is to divide the reads of each miRNA by the total number of reads mapping to the genome or to the known miRNAs. Methods developed for microarray normalization (e.g., lowess or quantile normalization) have also been applied to miRNA-seq data.

However, it is noteworthy that all global normalization methods (independently of the platform used) are based on the assumption that the same total amount of miRNAs is expected in all samples and that only a small percentage of miRNAs is differentially expressed, as both up- and downregulated. Such assumptions are often inappropriate for studies aimed to identify cancer-related circulating biomarkers, because in neoplastic patients, as already mentioned, mainly an increase in circulating miRNAs is expected compared with unaffected individuals.

This implies that whereas a general upregulation is present in raw data, not only due to technical biases to be removed but also due to the biological phenomenon under investigation, such a trend could disappear after application of classical normalization strategies. Such a

problem was clearly demonstrated in the case of an expected general miRNA downregulation as a consequence of inducible deletion of Dicer1. Consequently, although widely used in the literature, classical normalization methods are not suitable in many situations when analyzing circulating miRNA profiling data.

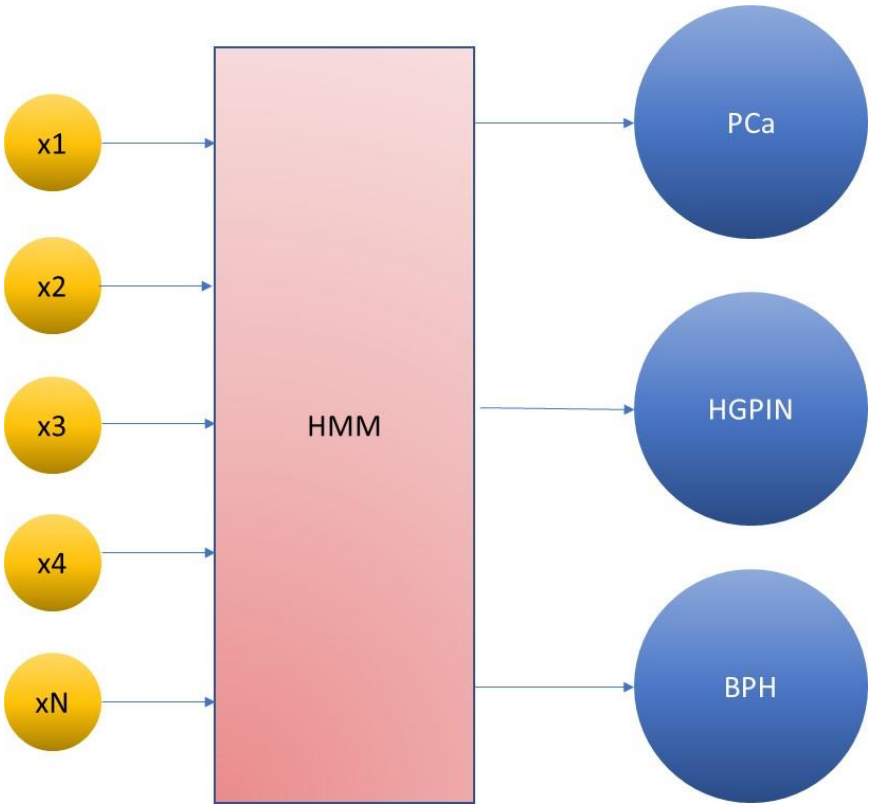
This implies that further research is needed to identify suitable methods, and, in this context, a ratio-based approach has been proposed, that is, like using, in turn, all miRNAs as housekeeping. Such an approach has the advantage of limiting a priori assumptions; however, high redundancy is generated in the data and it could become not trivial to establish which miRNA has a relevant role and which functions as a calibrator.

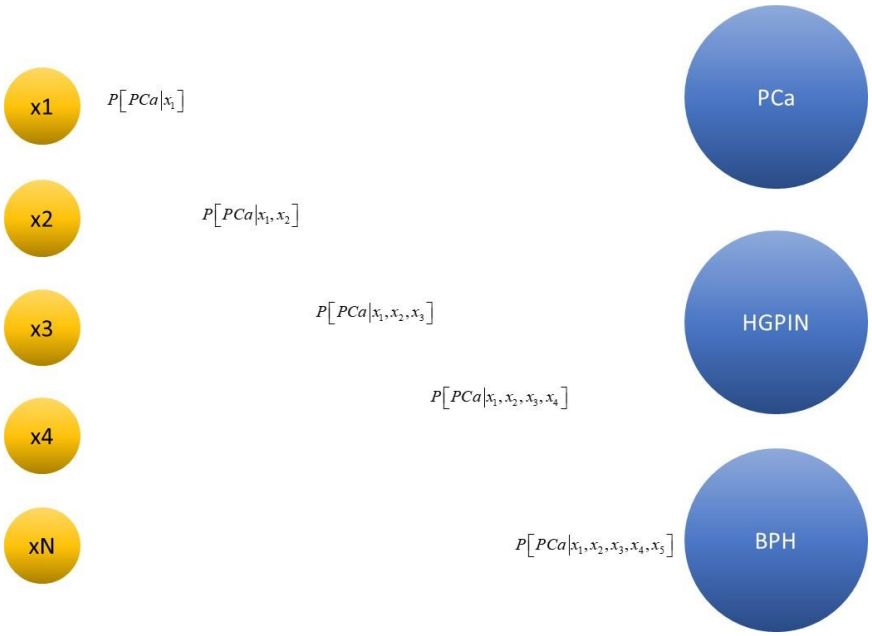
8.6 DISCRIMINANTS

We can now measure various miRNAs in body fluids and this gives rise to the liquid biopsy concept. However, the key question is how does one take a collection of miRNA measurements and ascertain, for example, that there is a prostate malignancy. For example we may from the previous presentation generate a vector of measurement of miRNA densities given by:

$$m_k = \begin{bmatrix} x_1 \\ \dots \\ x_n \end{bmatrix}$$

where this is for patient k and measures n miRNA densities. We want a discriminant function which takes these values and determines whether the patient has cancer of not. We could have a linear weighted discriminant or a more complex non-linear version.


x1
x2
x3
x4
xN
HMM
PCa
HGPIN
BPH


x1
x2
x3
x4
xN
$P[PCa|x_1]$
$P[PCa|x_1,x_2]$
$P[PCa|x_1,x_2,x_3]$
$P[PCa|x_1,x_2,x_3,x_4]$
$P[PCa|x_1,x_2,x_3,x_4,x_5]$
PCa
HGPIN
BPH

$$P\left[x_1 \middle| PCa\right]$$

....

$$P\left[x_1,...,x_N \middle| PCa\right]$$

or

$$P\left[PCa \middle| x_1\right]$$

....

$$P\left[PCa \middle| x_1,...,x_N\right]$$

where we have the two probabilistic ways to ascertain a condition based upon a data set.

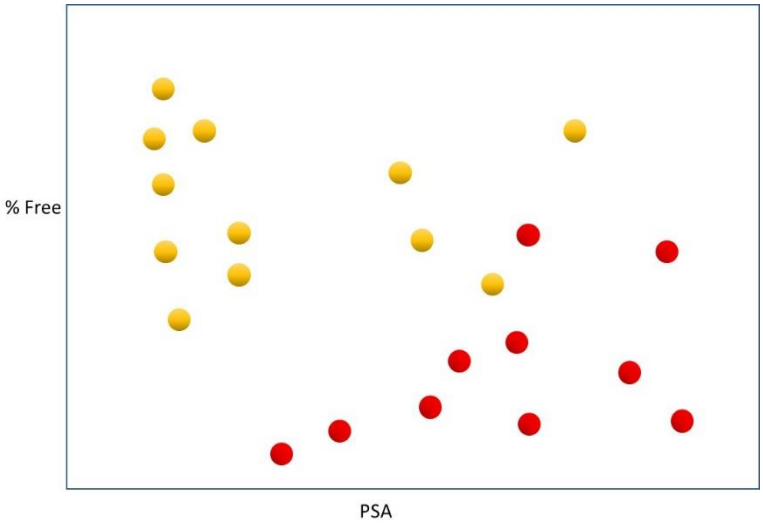
8.6.1 Simple Example

Let us consider a simple example. Assume we have to determine if a patient has prostate cancer or not. We are given three variables; PSA, % Free PSA, and PSA velocity[25]. Namely:

PSA=PSA
PF=% Free PSA

V=PSA Velocity

Thus we have three measurements and they are somewhat related. Let us start with two of them; PSA and PF. The data may appear as shown below:



[25] See: Carter et al, Detection of Life-Threatening Prostate Cancer With Prostate-Specific Antigen Velocity During a Window of Curability, Journal NCI Vol 98 Nov 2006 pp 1521-1527, https://academic.oup.com/jnci/article/98/21/1521/2521858

The red are PCa cells and the orange are benign. The higher the PSA the greater the chance for PCa. However, the higher the PF the greater the chance for benign, namely BPH. This is a simple case where we would have some discriminant where both variables count.

Now consider all three variables. We have PSA, PF and V. We need a discriminant so as to separate malignant from benign. We have data ex post facto so this is a supervised learning algorithm. We need to obtain some covering surface that maximizes the sensitivity and specificity. The algorithm must maximize the AUC. The more data the better the algorithm, yet we will always have aberrant cases.

The challenge in this case is that the discriminant is not a simple plane of some sort. It can be a complex surface winding its way around the 3-space. Namely the 2-space example shown in the above diagram may change for every V measure. For any V value we can obtain a 2-space profile. But that profile is different for every V and each has a different AUC. We can design a simple process where we enter all the data and calculate that surface on a cut by cut basis. Then any user can enter the three variable and get a result; benign/malignant, specificity, sensitivity.

Now let us consider a simple linear discriminant for PSA/PF and for a fixed V. Our goal is to select a curve:

$$PF = aPSA + PF_0$$

The goal is to obtain "a" and PF_0 so that we maximize both sensitivity and specificity. This can be readily accomplished by a variety of simple algorithms.

The next question would be; how many data points do we need and how frequently must they be updated? The answer can really only be obtained in an iterative manner with real data. We know that PSA alone has at best an AUC of 70%. Obtaining the AUC in this three element case is more complex. We may also want to add such elements as age, family history, prior biopsy results and the like. Each element adds another layer of complexity.

8.6.2 Complex Example

Now how do we apply this to miRNA measurements. Instead of readily understood parameters we have a complex set of somewhat misunderstood parameters. We do not know if they are correlated and, in fact, they may be representative of a multiplicity of malignancies as well.

Now consider the following list:

Target	***Organ***	***Target***	***Organ***
let-7a	Prostate	miR-199a-3p	Gastro
miR-1	Thyroid	miR-200a	Prostate
miR-106a	Prostate	miR-200c	Prostate
miR-106b	Prostate	miR-203	Thyroid
miR-10b	Melanoma	miR-206	Thyroid
miR-12	Thyroid	miR-200b	Prostate

Target	*Organ*	*Target*	*Organ*
miR-120	Prostate	miR-20a	Prostate
miR-124	Thyroid	miR-20a	Gastro
miR-124b	Prostate	miR-21	Prostate
miR-124a	Thyroid	miR-21	Gastro
miR-125b	Thyroid	miR-21	Thyroid
miR-126	Melanoma	miR-210	Gastro
miR-129	Thyroid	miR-214	Melanoma
miR-130a	Thyroid	miR-221	Prostate
miR-130b	Prostate	miR-221	Gastro
miR-133	Thyroid	miR-222	Prostate
miR-134	Thyroid	miR-223	Prostate
miR-135a	Prostate	miR-24	Prostate
miR-135b	Gastro	miR-24	Thyroid
miR-139-3p	Gastro	miR-26a	Thyroid
miR-141	Prostate	miR-28-3p	Melanoma
miR-142-5p	Melanoma	miR-28-5p	Melanoma
miR-143	Melanoma	miR-290	Thyroid
miR-143	Melanoma	miR-29a	Gastro
miR-144	Gastro	miR-29b	Gastro
miR-145	Prostate	miR-301b	Prostate
miR-145	Melanoma	miR-342-3p	Melanoma
miR-146a	Prostate	miR-342-5p	Melanoma
miR-146b-5p	Melanoma	miR-375	Prostate
miR-148a	Prostate	miR-378	Prostate
miR-150	Melanoma	miR-429	Prostate
miR-155	Gastro	miR-431b	Gastro
miR-155	Thyroid	miR-433	Prostate
miR-155	Melanoma	miR-451	Prostate
miR-15b	Prostate	miR-455-3p	Melanoma
miR-16	Prostate	miR-497	Melanoma
miR-17	Thyroid	miR-506	Gastro
miR-181	Thyroid	miR-561a-3p	Prostate
miR-183	Gastro	miR-605	Prostate
miR-18a	Gastro	miR-622	Gastro
miR-193a-3p	Melanoma	miR-9	Prostate
miR-193b	Melanoma	miR-9	Thyroid
miR-195	Prostate	miR-92a	Gastro
miR-196b	Gastro	miR-93	Prostate

Notice the many miRNAs which are reflected in different cancers. They do not dominate but they most likely should not be included since they can confuse.

Now we consider a subset for the purpose of diagnosing. We choose the following:

Target	***Source***	***Use***	***Organ***	***Details***
miR-16	Plasma	Diagnostic	Prostate	increased expression associated with high-grade (Gleason 8–10) disease
miR-130b	Plasma	Diagnostic	Prostate	Part of miRNA signature to distinguish PCa and BPH
miR-195	Plasma	Diagnostic	Prostate	increased expression associated with high-grade (Gleason 8–10) disease
let-7a	Whole blood	Diagnostic prognostic	Prostate	Decrease in PCa vs BPH; decreased expression with increasing D'Amico risk stratification
miR-93	Serum	Diagnostic prognostic	Prostate	increase in cancer vs healthy. Higher in high risk vs low and intermediate risk
miR-141	Whole blood	Diagnostic prognostic	Prostate	increase in PCa vs BPH. increased expression with increasing D'Amico risk stratification. Expression decreased after radical prostatectomy
miR-451	Serum	Diagnostic prognostic	451	increase in high risk vs healthy; no change between healthy, low-, and intermediate-risk

There are seven miRNAs in this set. We chose them solely on the basis of reasonableness. They are whole blood and serum and they are both diagnostic and prognostic. Thus to generate a decision space we would take this data from a large cohort of patients who are non-PCa and PCa. However we would most likely do so for early stage PCa so as not to confound the result. The intent is to find a decision algorithm which would maximize the sensitivity and specificity of the test algorithm. This is a classic example of a supervised learning algorithm. What we saw above was a similar example. Note also in the selection above we have chosen miRNAs which have been identified for Prostate only.

A simple and direct approach would be a linear classifier. Our metric is sensitivity and specificity. Namely:

$$Sensitivity = P\left[H_1 \middle| H_1 \right]$$

and

$$Specficity = P\left[H_0 \middle| H_0 \right]$$

If the discriminant plane is:

$$g(x) = ax + b$$

where

$$x = \begin{bmatrix} x_1 \\ \dots \\ x_N \end{bmatrix}$$

$$a = \left[a_1 \dots\dots a_N \right]$$

The goal is given the data set, find the **a** vector and b to separate the data so as to maximize sensitivity and specificity[26].

[26] We refer to Theodoridis and Koutroumbas and their work on classification. We note that there are a multiplicity of algorithms to define this linear classifier. Also, there is a great deal on PCa learning algorithms in Hastie et al.

There are a multiple set of classifiers and our selection of a linear classifier in a supervised environment is just one of many. We do not know the underlying statistics of the miRNA and also each miRNA itself may or may not be as strong an element in classification. Some miRNA that we choose may be a weak element and should be eliminated. That can only be ascertained after extensive data analysis.

Another way one could examine this partition problem is to assume that the two variables we discussed earlier, say PSA and PF, are independent Gaussian variable with mean and standard deviations:

$$H_0:$$
$$m = m_0, \sigma = \sigma_0$$
$$H_1:$$
$$m = m_1, \sigma = \sigma_1$$

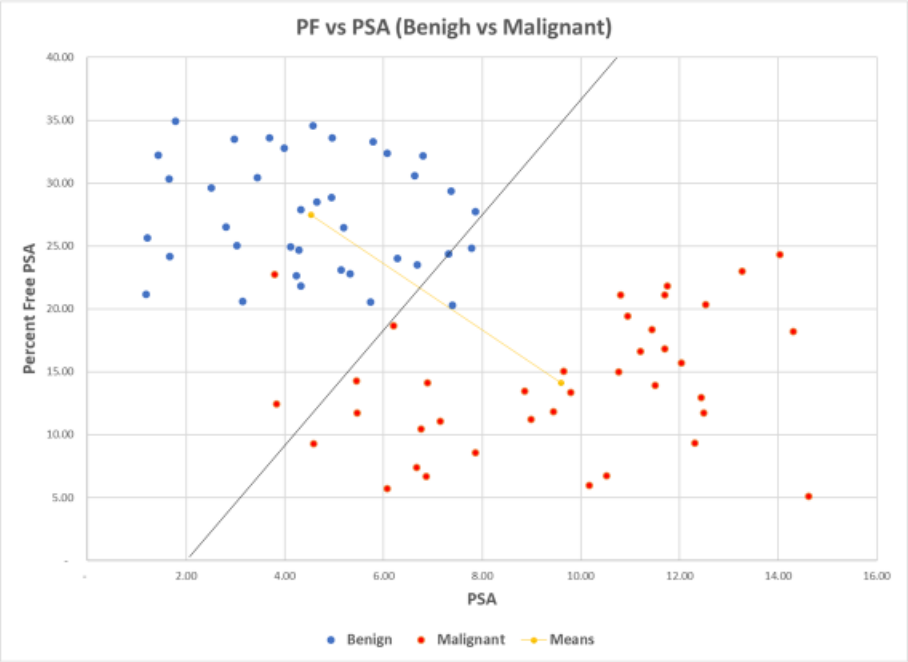
Then we could use classic decision analytical methods to determine optimal selection criteria. We could estimate the mean and variance from the given data and even ascertain a probability density function to see if it varies from Gaussian. It is not clear that such an approach yields better discrimination.

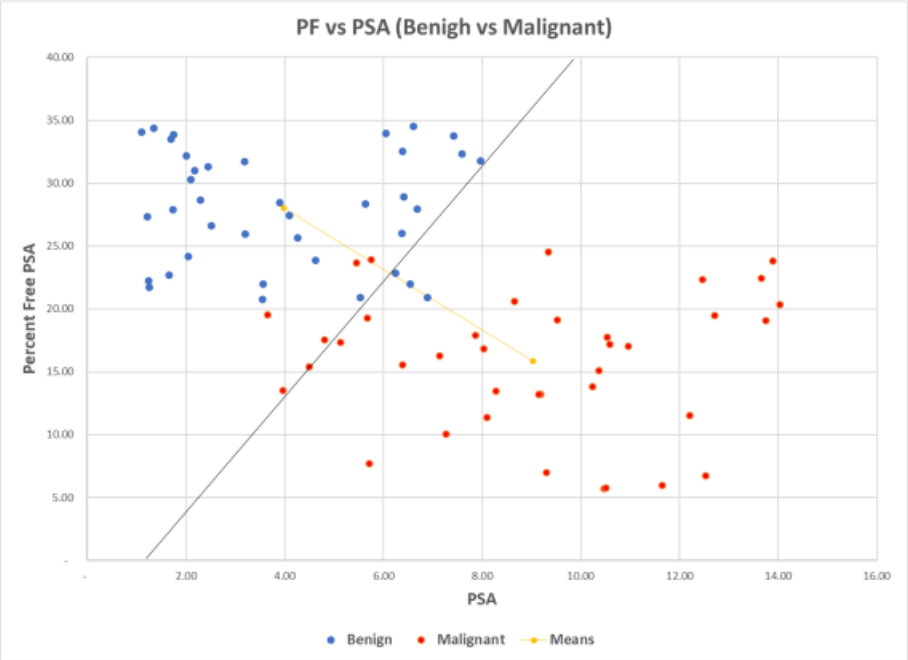
Finally, one could seek to use a Principal Component Analysis to determine optimal orthogonal axes[27]. However, again in my experience, this would not gain a great deal.

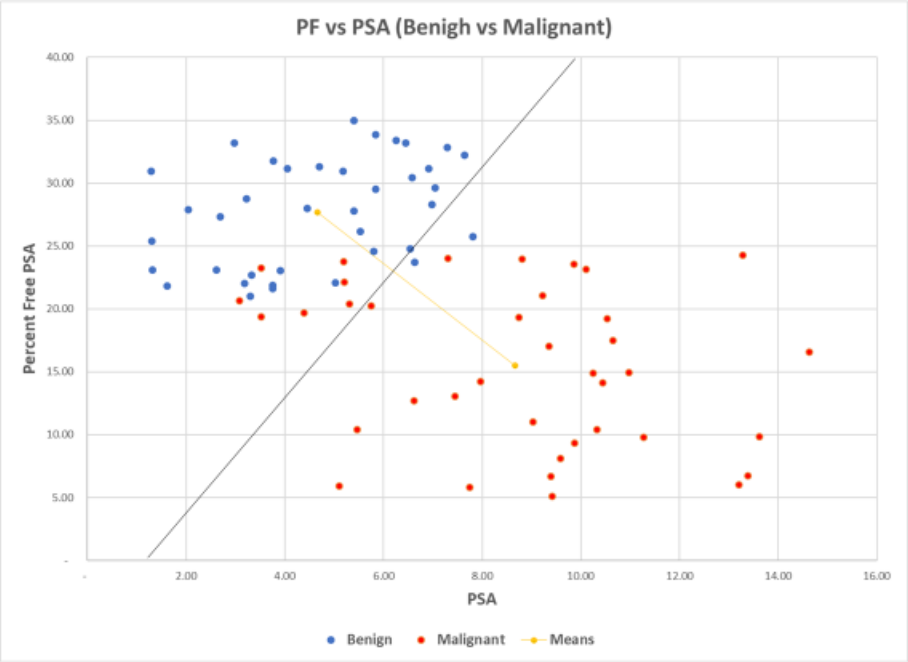
A linear classifier using the large data set may be more than adequate. We show below several examples of a linear classifier for PSA vs FP[28].

[27] See Dunteman, Principal Component Analysis, Sage University Paper, 1989.

[28] We use the reference of Duda and Hart, Pattern Classification, 1st Ed, Wiley, 1973.


PF vs PSA (Benigh vs Malignant)
Percent Free PSA
PSA
Benign
Malignant
Means


PF vs PSA (Benigh vs Malignant)
Percent Free PSA
PSA
Benign
Malignant
Means



Note all three have different data yet all three have same means on the two data sets. Thus the slope of the classifier is the same and intercept changes a bit. This same approach carries over to the miRNA context for multiple dimensions.

9 BIOMARKERS

Can miRNAs be used as biomarkers? We ask the general question and then is the answer is even that it is possible we can examine further. First let us examine just what we mean by a biomarker.

9.1 DEFINITION

Let us begin with a discussion of just what we mean by a biomarker[29]. From a recent discussion by Goosens et al we have the following discussion:

A biomarker is an objectively measured characteristic that describes a normal or abnormal biological state in an organism by analyzing biomolecules such as DNA, RNA, protein, peptide, and biomolecule chemical modifications.

However, it must be acknowledged that the definition of biomarkers has been evolving over the past decade, with one especially broad definition by the World Health Organization suggesting that "A biomarker is any substance, structure or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease."

More specifically in terms of clinical utility, a cancer biomarker may measure the risk of developing cancer in a specific tissue or, alternatively, may measure risk of cancer progression or potential response to therapy. Besides providing useful information in guiding clinical decision making, cancer biomarkers are increasingly linked to specific molecular pathway deregulations and/or cancer pathogenesis to justify application of certain therapeutic/ interventional strategies.

The conceptual framework of cancer biomarker development has also been evolving with the rapid expansion of our omics analysis capability of clinical biospecimens based on the traditional path of biomarker deployment. Cancer biomarkers can be classified into the following categories based on their usage.

***Predictive biomarkers** predict response to specific therapeutic interventions such as positivity/activation of HER2 that predicts response to trastuzumab in breast cancer. Similarly, KRAS-activating mutations predict resistance to EGFR inhibitors such as cetuximab in colorectal cancer.*

***Prognostic biomarker**, on the other hand, may not be directly linked to or trigger specific therapeutic decisions, but aim to inform physicians regarding the risk of clinical outcomes such as cancer recurrence or disease progression in the future. An example of a prognostic cancer*

[29] We have examined the issue of liquid biopsies earlier. See https://www.researchgate.net/publication/325023533_Liquid_Biopsy_and_Cancer

biomarker is the 21-gene recurrence score which was predictive of breast cancer recurrence and overall survival in node-negative, tamoxifen-treated breast cancer.

*Another class of biomarker, the **diagnostic biomarker**, is used to identify whether a patient has a specific disease condition. Diagnostic biomarkers have recently been implemented for colorectal cancer surveillance by testing for stool cancer DNA*

Thus we have at least the above classes. It is certain as we better understand the role of miRNAs this will expand.

9.2 MIRNA AS BIOMARKER

We have been giving a broad set of biomarkers for a variety of cancers. The intent is to demonstrate their usefulness but not to be a complete presentation. Many cancers were not discussed and our discussion of miRNA biomarkers was limited to select results. The full list of putative markers is significant and yet to be fully vetted. As Giza et al have noted:

Measurement of miRNAs in body fluids including plasma and serum may represent a gold mine for noninvasive biomarkers in cancer. The ultimate goal of molecular and computational approaches of the last decade in miRNAs research was to reveal the functions of miRNAs in human disease and to establish their role as biomarkers able to improve diagnosis and prognosis of disease. miRNAs can be considered early biomarkers due to their upstream positions in the regulation cascades.

Moreover, they can be easily identified using genomic tools such as oligonucleotide microarrays and deep sequencing which deliver higher throughput than mass spectrometry used for protein and metabolite biomarker identification. miRNAs represent a novel attractive diagnostic biomarker due to their higher stability when compared to RNAs and remain stable after being subjected to severe conditions that would normally degrade most RNAs, such as boiling, very low or high pH levels, extended storage, and 10 freeze-thaw cycles.

Even in low abundant expression, miRNA can be amplified and then detected in a clinical setting by real-time quantitative PCR (qPCR), an approach used in FDA-approved clinical tests already. The adoption of the locked-nucleic acid (LNA) technology in miRNA probe design could improve the sensitivity and specificity of miRNA qPCR assays even further. MiR-146a, miR-150, miR-223, miR-574-5p and a panel of 6 plasma miRNAs were found already to be potential biomarkers for sepsis and systemic inflammatory response syndrome (SIRS).

Recent published data clearly suggested that miRNAs profiling is useful in identifying predictive miRNA signatures associated with several cancer types like pancreatic cancer, colorectal cancer, cell renal cell carcinoma, and osteosarcoma. The changes in miRNAs expression can be also detected from circulating tumor cells in blood samples/ plasma and urine or by tumor slide-based staining.

Furthermore, correlations between circulating miRNA expression levels and response to a given anticancer treatment have already been made. Serum miR-21 levels were higher in hormone-

refractory prostate cancer patients whose disease was resistant to docetaxel-based chemotherapy when compared to those with chemosensitive disease

There are many miRNAs as we have noted that appear again and again in multiple cancers. They in a sense become the p53 type element where we can say that on the one hand and then on the other. No single miRNA seems to be a unique marker. It is expected as we have discussed previously that a profile of a set of such miRNAs may apply to individual cancer variants.

As Tiberio et al have noted:

In the last few years, there has been increasing interest in circulating miRNAs as cancer biomarkers, due to their high stability, their putative capability to be more informative than mRNA, and the noninvasiveness of their detection. Since their discovery in body fluids, considerable effort has been directed to investigate the relevance of these small RNAs in different diseases, and now there is much evidence of their potential clinical relevance as cancer biomarkers in different types of malignancies.

The first study that identified specific circulating miRNAs associated to cancer was by Lawrie et al., who in 2008 found high levels of miR-155, miR-210, and miR-21 in patients with diffuse large B-cell lymphoma and demonstrated a significant correlation between high levels of miR-21 and relapse-free survival. A literature survey concerning the principal findings related to the usefulness of specific circulating miRNAs (or miRNA signatures) as diagnostic, prognostic, and/or predictive parameters in different cancer entities has…

Plasma or serum miRNAs appear to display a promising potential mainly in the diagnosis of different solid tumors at preoperative level, thus suggesting the possibility of their utility as early-diagnostic tools. Accordingly, a very recent study performed in a large cohort of smoker individuals provided evidence that specific ratio-based miRNA signatures (including 24 distinct miRNAs assayed in plasma samples) have significant diagnostic and prognostic power to anticipate the detection of malignant lung cancers and to predict tumor aggressiveness…..

It should be pointed out that circulating miRNAs might not only represent promising noninvasive diagnostic and prognostic tools but they could also be used to predict and monitor the efficacy of anticancer treatments. In this context, recent correlative studies within neoadjuvant or adjuvant chemotherapy trials identified many circulating miRNAs as associated with response to treatment and drug resistance.

For example, in HER2-positive breast cancer patients undergoing neoadjuvant therapy, plasma miR-210 levels were found to be associated to trastuzumab sensitivity, thus suggesting that plasma miR-210 levels might be used to predict and monitor response to therapies containing the monoclonal antibody. In the context of adjuvant chemotherapy regimens, it has been shown that serum miR- 21 levels can predict the benefit of gemcitabine treatment in advanced pancreatic cancer patients, suggesting that the miRNA might be used as a predictor of the chemosensitivity to this nucleoside analogue.

However, despite the fact that several published papers demonstrated the feasibility of using circulating miRNAs as putative cancer biomarkers, many preanalytical and analytical aspects,

as well as donor related factors, can interfere with accurate circulating miRNA quantification, and future studies have to take them into consideration.

Preanalytical and Analytical Variables Affecting Circulating miRNA Studies In a recent work by Leidner et al., the authors highlighted for the first time the widespread inconsistency across circulating miRNA studies, cautioning the scientific community about the huge variety of methodological parameters impairing circulating miRNA evaluation. …summarizes the main preanalytical and analytical variables interfering with circulating miRNA analysis that are examined in detail in the following sections.

For example, in melanoma Gajos-Michniewicz and Czyz have noted the following biomarker importance:

There is a great need for new diagnostic tools to detect melanoma, especially at the early curable stages of development. miRNAs have great potential as biomarkers because they can discriminate among diverse types of cancers and they are chemically stable and resistant to RNase activity.

*Fleming et al., have demonstrated that the assessment of four miRNAs (**miR-15b, miR-30d, miR-150, and miR-425**) has a greater potential of predicting melanoma recurrence than TNM staging.*

*Shiiyama et al. identified six miRNAs (**miR-9, miR-145, miR-150, miR-155, miR-203, and miR-205**) that were differentially expressed in metastatic melanoma patients compared to healthy donors, and their combination was more sensitive for detecting metastasis than each miRNA assessed individually.*

*The comparison of miRNAs from melanoma tissue samples with matching serum samples revealed that several miRNAs (e.g., **miR-221, miR-222, and miR-3201**) are only present at high levels in serum, whereas others are exclusively tissue e-derived (e.g., miR-30 and miR-374). As melanoma survival rates are higher when the cancer is diagnosed early, non-invasive liquid biopsies would be an optimal source of biomarkers that indicate disease development.*

This approach may also provide an opportunity for disease monitoring during treatment. In this regard, miRNAs are a long-investigated component of the circulating transcriptome.

*In a recent study, 11 miRNAs (**let-7b, miR-16, miR-21, miR-92b, miR-98, miR-134, miR-320a, miR-486, miR-628, miR1180, and miR-1827**) were identified as differentially expressed between healthy controls and plasma samples from different melanoma stages. Two miRNAs (miR-320a and miR-134) have been found at lower levels in plasma from melanoma patients compared to samples from healthy donors.*

*miR-21 has been reported at elevated levels in melanoma plasma samples. Eight miRNAs (**let-7e, miR-99b, miR-100, miR-125a, miR-125b, miR-146a, miR-146b, and miR-155**) have been detected in patients receiving the ICIs ipilimumab and nivolumab as correlated with the frequency of altered myeloid cells and shorter PFS (progression-free survival) and OS (overall survival).*

Additional studies are needed for a more critical evaluation of the clinical value of miRNAs, and the use of miRNAs' expression patterns as reliable biomarkers capable of detecting primary disease and early metastasis after treatment. From the perspective of therapeutic applications, strategies to deliver tumour-suppressive miRNAs or interfere with tumour-promoting miRNAs are still under development

As noted above, there are a variety of possible markers. It will be a challenge to assemble these into a simpler set. On the otherhand, perhaps individual miRNA profiling may be more useful.

10 OBSERVATIONS

Let us now consider just what we would examine and how to interpret the measurement. Let us consider some sample of accepted markers. First consider the PSA test. We know that it is organ specific, it relates to the prostate. Second we can measure it in a somewhat consistent manner with assays. It should be noted that there is an inter-assay variance. Third it is not a perfect test with an AUC of less than 80%. Fourth it is readily administered. Fifth, and this is critical, it is one measurement.

What do we measure when we measure miRNAs? Let us consider a list:

1. For each miRNA we can measure a relative intensity. We cannot measure the mg/L or some other well-defined number. We can say it is about x times some standard we attach it to.

2. It is not dispositive. Take miR-21. There may be a lot of organs from which it comes and there are a variety of simple or complex reasons for its measure. Thus we do not necessarily measure the result from a specific organ.

3. There are many factors impacting the expression of miRNAs. Thus without fully understanding these co-factors we may very well establish inaccurate results.

Thus, what we measure can be a complex "image" and not a simple number. The "image" may be a map of intensities of a set of miRNAs measured. There may be N such measurements and each brings to the process information but not complete information.

10.1 THERAPEUTICS

From Xie et al:

MicroRNAs and long noncoding RNAs have long been investigated due to their roles as diagnostic and prognostic biomarkers of cancers and regulators of tumorigenesis, and the potential regulatory roles of these molecules in anticancer therapies are attracting increasing interest as more in-depth studies are performed. The major clinical therapies for cancer include chemotherapy, immunotherapy, and targeted molecular therapy.

MicroRNAs and long noncoding RNAs function through various mechanisms in these approaches, and the mechanisms involve direct targeting of immune checkpoints, cooperation with exosomes in the tumor microenvironment, and alteration of drug resistance through regulation of different signaling pathways. Herein we review the regulatory functions and significance of microRNAs and long noncoding RNAs in three anticancer therapies, especially in targeted molecular therapy, and their mechanisms.

The authors then delineate a set of targets which are currently under examination.

Cancer Type	*ncRNA*	*Regulation of Chemoresistance*	*Target*
NSCLC	miR-197	promotion	CKS1B/STAT3
NSCLC	miR-130b	promotion	Wnt/b-catenin pathway
Prostate	miR-455-3p	promotion	TAZ
Prostate	miR-29c	inhibition	USP22
Prostate	miR-374b-5p	inhibition	Breastl-2
Breast	miR-503	inhibition	CCND2, CCND3
Breast	miR-17	promotion	DEDD
Stomach	miR-218	inhibition	mTOR inhibitor
Stomach	miR-623	inhibition	CCND1
Colon	miR-191	promotion	Wnt/b-catenin pathway
Colon	miR-519b-3p	inhibition	ARID4B mRNA
Colon	miR-15	inhibition	NF-kB, Breastl-2
Esophageal	miR-125a-5p	inhibition	STAT3
Liver	miR-16	inhibition	NF-kB
Ovary	miR-630	promotion	APAF-1
Ovary	miR-142-3p	inhibition	Sirtuin 1

10.2 STEM CELLS

Over the years we have examined the stem cell paradigm for various cancers. It is interesting to see this get integrated into the discussion on miRNAs. As Liu and Tang have noted:

Research in the past decade suggests the presence of cancer stem cells (CSC) that can both regenerate themselves and differentiate into a spectrum of maturing daughter cells, which create the cellular heterogeneity of cancer. CSCs were first discovered in acute myeloid leukemia and, since 2003, have also been reported in most solid tumors.

Emerging evidence indicates that CSCs may be involved in tumor maintenance, therapy resistance, tumor progression, and distant metastasis. Despite their potential clinical significance, how intrinsic CSC properties are regulated at the molecular level is poorly understood. Recent discoveries of microRNAs (miRNA) have provided a new avenue in understanding the regulatory mechanisms in CSCs. miRNAs are 21- to 25-nucleotide (nt)–long, noncoding RNAs that induce the target mRNA degradation or repress mRNA translation by imperfect binding to their 30-untranslated region .

The miRNA gene is first transcribed by RNA polymerase II into primary transcript (pri-miRNA) in the nucleus, where the hairpin stem-loop structure is processed into precursor miRNA (pre-miRNA) by a microprocessing complex, including Drosha and DGCR8. The 70-nt-long pre-miRNA is then exported into cytoplasm, where it undergoes a second processing by Dicer, in

which one strand of the hairpin is incorporated into the ribonucleoprotein complex called miRNA-induced silencing complex. A single miRNA may target dozens of mRNAs, and one mRNA can be regulated by multiple miRNAs.

Although small, miRNAs play a powerful role in biological processes including development, proliferation, and apoptosis. Early studies have linked miRNAs to controlling the self-renewal and differentiation of embryonic stem cells (ESC), and later, aberrant expression and/or functions of miRNAs are implicated in tumorigenesis. More recent studies suggest that miRNAs may also regulate CSC properties.

10.3 MEASUREMENT ISSUES

Measuring miRNAs is currently a time consuming, costly, and somewhat error prone process. The PCR approach lends itself to measuring intensity of individual miRNAs, yet this as we have discussed has its limitations. As Thind and Wilson have observed:

Current miRNA measuring techniques also suffer from problems that limit their clinical effectiveness. Two commonly used methods for measuring ex-miRNA in research are microarray and reverse transcription polymerase chain reaction (RT-PCR).

Microarray offers genome-wide expression profiles of miRNA, facilitating the detection of a large number of aberrant miRNAs. However, there is difficulty developing probes for some miRNAs, and the same hybridisation conditions do not work for all molecules. Compared to RT-PCR, microarray has higher specificity but a lower sensitivity. Despite similar accuracy, microarray has been shown to be unsuitable for quantifying low levels of miRNAs and thus would require larger sample sizes for quantification.

Microarray is an older mechanism and its limitations are significant. It is easier but lacks the ability to measure intensity.

However, RT-PCR requires a suitable ‘‘housekeeping’’ miRNA control, which is often not reliable. A frequently used control is miR-16, but it is dysregulated in myeloma and rheumatoid arthritis.

Absolute qPCR overcomes this issue, although a standard calibration curve for specific miRNAs always needs to be constructed and consistently high-quality exosome preparations are needed. The accuracy of these methods could be limited further by the presence of exosomal mRNA and premiRNAs that may also be measured within isolated exosomes.

Additionally, miRNAs have been identified to exhibit ‘‘isomiRs’’ (sequence heterogeneity at the 3’ and 5’ ends) that may further complicate measurements, particularly for qPCR-based methods. A more recently adopted method for measuring expression profiles of miRNA is next-generation sequencing, a powerful approach that may prove useful clinically.

This method enables accurate genome-wide quantification of miRNAs and can distinguish between miRNAs that differ by even 1 nucleotide. Since no primers or probes are required, it can

detect novel miRNAs. Nevertheless, data analysis and sample preparation are labour intensive and require personnel expertise

Quantifying we believe is a critical factor in determination for usefulness. Yet this, as noted, is reliant upon personal skill. Perhaps this may be mitigated by automatic techniques but none are yet apparent.

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