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THE LONG AND WINDING ROAD OF MICRORNA PROFILING FROM BENCH TO BEDSIDE

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Abstract – *MicroRNAs (miRNAs) are short non-coding RNAs that control the expression of the majority of protein-coding genes within a cell. Their dysregulation is both cause and indication of a variety of diseases, including cancer. Specific patterns of miRNAs' expression enable researchers to classify cancer subtypes and their differentiation grade. As a consequence, miRNAs are considered promising cancer biomarkers to be adopted in the clinical routine.*

Recent technical advancements have allowed for the profiling of thousands of miRNAs in parallel, and in large sets of samples. However, the information gained from these analyses has been insufficient to build reliable diagnostic and prognostic workflows in the clinical laboratory.

Here we describe some of the recent protocols used to profile miRNA expressions and highlight both advantages and limitations associated with them. Finally, we propose that, besides the measurement of total expression of miRNAs, assessment of other parameters–such as miRNAs' editing patterns and subcellular localization–may be integrated to make miRNAs more powerful cancer biomarkers.

KEYWORDS: *Cancer biomarkers, Circulating microRNAs.*

INTRODUCTION

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the post-transcriptional level by inhibiting the translation of target mRNAs, and by inducing their degradation[1].

The first phase of miRNA biogenesis is the transcription of a long primary miRNA (pri-miRNA), which is rapidly cleaved in the nucleus by the RNase III Drosha, as part of a large multi-subunit complex named the microprocessor[2]. The resulting product, the precursor miRNA (pre-miRNA), is exported to the cytoplasm by the exportin 5 complex, wherein it undergoes a second cleavage step operated by the RNase III DICER[3]. The product of DICER catalysis is a short double-stranded RNA, which is loaded into one member of the Argonaute (AGO1-4) protein family.

On AGO, typically, one RNA strand is degraded, whilst the remaining is the final mature miRNA. Following this, the miRNA-loaded AGO associates with one of the members of the TNRC6 protein family to form the core of the miRNA-Induced-Silencing Complex (RISC), the effector machinery that mediates miRNA function[4].

The partial complementarity between the sequence of miRNA, and sites on the 3'UTR of the mRNAs is the basis of the mechanisms of targeting; The miRNA-loaded unit, finds its cognate binding site on the mRNA, with consequential recruitment, through TNRC6, of de-adenilases and de-capping proteins that ultimately make the mRNA substrate for exonucleases in the cell[5,6].

More than 2600 miRNAs have been discovered in human to date[7]. Each miRNA can target hundreds of different mRNAs; as a result, about 60% of the transcriptome is under miRNA-mediated repression.

Due to this, it is not surprising that miRNAs control essentially every biological process within a cell, and their dysregulation is both cause and indication of a variety of diseases, including cancer[8].

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MIRNA EXPRESSION PROFILING IN CANCER

The interest in profiling miRNA expression stems from seminal reports showing that the majority of miRNAs reside in cancer-associated genomic region and fragile sites[9], therefore indicating that they could act as tumor suppressors. However, soon after, it became clear that miRNAs could function both as tumor suppressors and oncogenes, as in some cancers are found down regulated or lost, and in others are found overexpressed.

The optimization of high throughput platforms, such as next generation sequencing (NGS) and microarrays, provided the opportunity to determine miRNA expression profiles in large sets of tumor samples, showing that specific signatures of miRNA expression are able to reveal cancer subtypes, as well as their differentiation stage[10]. Thereafter, miRNAs started to emerge as a promising new class of cancer biomarkers.

To reinforce the interest in miRNA profiling, it was the discovery that, in addition to being expressed inside cells and tissues, miRNAs are present in detectable amounts in bodily fluids such as peripheral blood, urine, saliva, and breast milk[11]. The discovery of miRNAs in these fluids represented a clear operational advantage as they can be sampled by non-invasive procedures. Moreover, they are the standard sample types processed in clinical laboratories; as a result, they can be easily integrated into pre-existing workflows.

Of particular interest are blood borne miRNAs, which are detectable in both serum and plasma and are referred to as circulating miRNAs[12,13]. Circulating miRNAs originate from multiple tissues and blood cells[14-16], and can be released as exosomes, microvescicles, or apoptotic bodies[17,18].

The presence of significant amounts of miRNAs in the serum and plasma can be explained by their remarkable stability, conceivably due to their small size. In fact, being only about 22-nt long, miRNAs can be embedded within AGO proteins, which protect them from degradation by exonucleases.

Therefore, circulating pool of miRNAs represents the most promising candidate for the early detection of cancer-related miRNA signature.

METHODOLOGIES TO PROFILE MIRNAS

Several promising assays designed to detect miRNAs in biological samples exist[19-23]. However, the challenge is to bring these technologies to routine clinical applications.

For clinical purposes, the ideal methodology requires the following characteristics:

1) The methodology should present high sensitivity, to allow quantitative analysis even in samples containing low number of copies of the target miRNA.
2) Single-nucleotide differences must be captured, as miRNAs can differ by as few as one nucleotide.
3) High reproducibility is required to allow statistical significance and validation for a clinical workflow.
4) Methodologies should be adaptable to automated systems in order to allow processing of multiple samples in parallel within a clinical lab.

As each of the current methodologies presents both strengths and disadvantages, identifying a gold standard for miRNA analysis is complicated. Instead, the choice surrounding which methodology to adopt should be driven by the experimental needs, such as sample source, and expected amount of miRNAs to be detected in the sample.

RT-QPCR

Due to its high sensitivity and specificity, to date, RT-qPCR is the most used technology for miRNA quantification[24-28].

Given their short length of miRNAs, it has been a challenge to design specific primers to reach reliable amplification signals. Indeed, miRNAs are basically the length of a single PCR primer. To allow the binding of the primers, extension of the target miRNA before performing qPCR is required. To this end, two main strategies are currently adopted: one uses a stem-loop RT primer, the other a poly-A tailing followed by RT with an oligo-dT primer.

In the stem-loop RT primer approach[29] a looped primer is used to initiate the RT reaction after hybridization to the 3'-end of the miRNA. The stem-loop primer is formed by a miRNA-specific region complementary to the 3'-end of the miRNA, and by a 5'-end universal region. Although the pairing between primer and miRNA is only a few bases, sufficient free energy for effective binding is achieved, as the base stacking of the stem enhances the stability of miRNA-DNA heteroduplexes. Once the primer is extended, the resulting cDNA can be amplified with a miRNA-specific forward primer and a universal reverse primer, complementary to the universal region of the stem-loop RT primer.

Other qPCR methods use poly-A tailing to lengthen the miRNA[30]. Poly(A) Polymerase (PAP) catalyzes the transfer of adenosine from ATP to the 3'-end of any RNA. PAP is used to create a poly-A tail at the 3'-end of the miRNA, and then retrotranscription can be primed using an oligo-dT primer. The oligo-dT primer includes a universal sequence at its 5'-end, which enables subsequent qPCR using

a miRNA-specific forward primer and a reverse primer that is complementary to the universal sequence.

ISOTHERMAL AMPLIFICATION

Isothermal amplification represents a valuable alternative to PCR-based techniques for the amplification of nucleic acids, and it has been applied to the detection of miRNAs[31]. The advantage of this technique compared to PCR is that the amplification is carried out at constant temperature; therefore, expensive thermal cycler systems are not required. Several variants of this technique have been developed to successively detect miRNAs, such as rolling cycle amplification (RCA[32]) exponential amplification reaction (EXPAR[33]), and loop-mediated isothermal amplification (LAMP[34]). Because basic lab equipment is required, these systems are considered extremely promising, especially for "point of care" applications.

NORTHERN BLOTTING

Northern blotting (NB) was one of the first techniques used to measure the expression levels of individual miRNAs[35]. In traditional NB analyses, total RNA is size-separated through polyacrylamide gel electrophoresis, transferred to a membrane, and hybridized with a radio-labeled DNA probe complementary to the target miRNA. However, although NB provides information regarding both sequence and length of the miRNA, due to associated health hazard concerns, and the special training required to perform this technique, its usage is not suitable in a clinical setting. However, NB still represents a valuable approach in basic research.

LATERAL FLOW ASSAY (LFA)

(LFA)-based systems are versatile tools for molecular detection[36]. In these assays, a liquid sample containing the target molecule moves by capillarity along a polymeric strip, on which molecules that can bind to the analyte are attached. The sample is applied at one end of the strip, which is soaked with buffer to ensure proper flow, and then migrates until it reaches a detection zone, where specific antibodies or a sequence-specific capture nucleic acid probe is immobilized. Antibodies are usually conjugated to chromogens or fluorescent moieties, whose reaction with the analyte results in a signal emission that can be quantified by a standard strip reader instrument.

Hou et al. have employed LFA-based approach using a DNA probe conjugated with gold nanoparticles for visual detection of miRNA-21[37]. In this system, the signal emitted from the labeled probe is maintained only if the target miRNA is present in the sample, as a single-strand-specific nuclease catalyzes the degradation of the capture probe if there is no target miRNA in the samples. This technique presents many advantages due to its low cost, simple instrumentation needed, and its ability to detect target miRNA at the attomole scale.

OLIGONUCLEOTIDE-TEMPLATED REACTIONS (OTRS)

OTRs are another effective tool to detect nucleic acids in biological samples. In this system, two probes conjugated with reactive groups come to close proximity when they bind to a target nucleic acid sequence. As a result, the presence of target sequence in the sample can be detected by the production of a signal resulting from the chemical reaction between the two reactive groups. Metcalf et al. have recently described a novel strategy based on OTRs, by which they were able to successfully quantify circulating miR-375, miR-141, and miR-132 in blood samples[38].

NANOBEAD-BASED SYSTEMS

In these systems, a single strand DNA probe, labeled with a fluorophore and with sequence complementary to the target miRNA, is immobilized on PEGylated gold nanoparticles (AuNPs). When the fluorophore is in proximity of the nanoparticle, fluorescence is inhibited. However, upon hybridization of miRNA with the probe, the resulting DNA-RNA heteroduplex becomes substrate for a duplex-specific nuclease, which selectively cleaves the DNA strands, thus releasing and disinhibiting the fluorophore. As a result, fluorescence emission is proportional to the number of miRNA molecules in the sample. It has been shown that this assay is able to quantify miRNAs at the sub-femtomolar scale[39].

DNA NANOSWITCHES

Recently, a non-enzymatic miRNA detection assay, named miRacles, has been developed[40]. This assay employs conformationally responsive DNA nanoswitche. DNA nanoswitchs are linear double-stranded DNA segments that forms a loop in the presence of a target miRNA. The nanoswitch are generated by hybridizing short oligonucleotides complementary to long single-stranded DNA. Two distant detector strands are designed to contain overhangs complementary to different segments of the target

miRNA. The binding of the miRNA to the detector induces the folding of the double strand segment, which shifts from the linear "off" state to the looped "on" state. The two states have different mobility in a standard agarose gel electrophoresis; therefore, they can be easily identified and quantified. This assay is able to distinguish miRNAs that differ from just one nucleotadide and can be performed in less than one hour using basic lab equipment.

IN SITU HYBRIDIZATION (ISH)

Although sensitive, the above-mentioned techniques do not allow for the retention of cyto-architectural details of the histological specimen, which are extremely informative for cancer diagnosis.

Historically, ISH has been optimized to allow precise detection and localization of specific nucleic acids within a histological section, while preserving visuo-spatial information in tissue samples[41]. The principle of ISH is that the target sequence within the histological specimen can be detected through the hybridization of a complementary nucleic acid probe conjugated with a reporter. Renwick et al[42] have developed a multicolor miRNA FISH protocol that allows the visualization of multiple miRNA in parallel in formalin-fixed paraffin-embedded (FFPE) tissues. By using this method, the authors were able to discriminate between a basal cell carcinoma (BCC) and a Merkel cell carcinoma (MCC), two skin tumors presenting overlapping histologic features but with distinct cellular origins. This work demonstrates the diagnostic potential of miRNA FISH in clinical laboratories.

HIGH THROUGHPUT METHODS

Microarray

Microarrays are widely used for gene expression analysis studies and are among the first technologies to be employed to identify miRNA expression signatures in cancer[43]. Microarrays principle lays on the hybridization between the target miRNA and complementary probes spotted on a chip. The advantage of microarray-based approaches is their ability to quantify large numbers of miRNAs simultaneously in a single test.

Next Generation Sequencing (NGS)

Sequencing is the most powerful approach for miRNA analysis[44]. Like microarray, NGS allows for the parallel quantification of known miRNAs, but in contrast to microarray–based approaches, it also allows the discovery of new ones. Moreover, it effectively allows for the discrimination of miRNAs that differ by as little as one nucleotide. However, some limitations are associated with this platform; first, NGS and microarrays are still too expensive for routine laboratory testing. Second, it requires computational power for data analysis and interpretation. Therefore, more technical advancements are necessary in order to bring these platforms into the clinical routine.

CHALLENGES IN USING MIRNAS AS BIOMARKERS

As mentioned, many systems to detect miRNAs have been developed and progress has been made. However, their employment in clinical routines has been a challenge.

For example, the New York State Department of Health Clinical Laboratory Evaluation Program has approved a molecular test developed by Rosetta Genomics for clinical use[45,46]. This test is able to classify squamous-cell carcinoma of the lung with a sensitivity of 96% and specificity of 90%. However, despite the enormous potential, adoption of these tests by the clinical practice still requires more clinical studies[47,48].

One of the reasons limiting the use of miRNAs as a diagnostic tool is the fact that dysregulation of a specific miRNA is not unique to a particular cancer type. For instance, the upregulation of circulating miR-21 has been found in patients with liver, lung, colorectal, breast and prostate cancer[49]. Additionally, the results are not consistent even when studies on the same diseases are evaluated[50].

The reasons for inconsistency may reside on the fact that, tumor burden and its localization in the body can differently induce systemic responses that may affect the secretion of miRNAs from distant organs. This is likely a potential confounding element responsible for the above-mentioned inconsistencies.

Extraction and analysis of circulating miRNAs also suffer from sampling limitations[51]. For example, during handling and storage of the sample, miRNAs may be released from blood cells and therefore affect the levels of miRNA in the plasma and serum[52]. Moreover, some anticoagulants used for sample collection inhibit the reverse transcriptase and DNA polymerase enzymes[53], thus affecting the overall signal and potentially skewing the analysis.

In conclusion, in order to use miRNAs as cancer biomarkers within the clinical laboratory, standard workflows must be designed. These workflows may encompass details surrounding sample collection,

miRNA isolation and quantification, and data analysis. Moreover, profiling the expression of a panel of selected miRNAs, as opposed to a single one, could be more effective in identifying cancer type-specific biomarkers.

NOT ONLY EXPRESSION: EXPANDING THE DIMENSIONS OF MIRNA ANALYSIS

Once transcribed, miRNAs undergo a series of processes that determine, not only their total amount within the cell, but also their final chemical structure, sequence, inclusion in multi-protein complexes, and subcellular localization. Can these additional properties be taken into account to refine better diagnostic and prognostic tools? An increasing number of experimental evidence indicates that the analysis of miRNA function is indeed a multi-dimensional approach, in which expression level is only one of the parameters that can be harnessed for diagnostic and prognostic purposes.

POST-TRANSCRIPTIONAL EDITING EVENTS

RNA editing is a post-transcriptional modification process that confers specific chemical changes at the RNA level. These chemical modifications ultimately result in changes in the sequence of the nucleotides in the RNA molecule, without affecting the corresponding sequence in the DNA[54,55]. The most common class of RNA editing in humans is the conversion of adenosine to inosine (A-to-I), which is catalyzed by the adenosine deaminase acting on RNA (ADAR) enzymes.

As such, miRNAs are also a substrate of A-to-I conversions[56]. Because the mechanism of miRNA targeting relies on the base pairing between miRNA and target mRNA, a single-nucleotide change can significantly alter miRNA target recognition[57].

Several miRNA editing events appear to be critical in cancer. For example, a single-base change due to the misregulation of the editing of miR-376a, alters the selection of its target mRNAs and redirects its function from inhibiting to promoting glioma cell invasion. This can be explained by the fact that the edited miRNA acquires the ability to target the AMFR transcript, and at the same time, loses its ability to inhibit the original target RAP2A[58]. In other words, A-to-I editing not only decreases the amount of the miRNA version corresponding to sequence of the gene, but also has the potential to generate a neomorphic variant that can repress a different set of targets.

Genome-wide analyses have shown that these miRNA editing events extensively correlate with clinical parameters, such as tumor subtype, tumor differentiation stage, and overall survival[59].

SUBCELLULAR LOCALIZATION

Several reports indicate that miRNAs are also localized within the nucleus of cells[60-62]. Importantly, while nuclear localization of miRNA is a widespread phenomenon, the relative abundance of miRNAs in the nucleus varies across tissue types. For example, miR-29b is enriched in HeLa cells[63] but not in other cell lines[64]. High-throughput profiling technologies have abundantly supported the existence of hundreds of nuclei-enriched miRNAs[65]. Free-access databases, such as RNALocate (http://www.rna-society.org/rnalocate/)[66], provide detailed information about miRNA subcellular localization. But what are the functions of nuclear miRNAs? The most described nuclear function of miRNA is its ability to bind to promoter regions. The interaction between miRNA and promoter is mediated by AGO proteins[67], and the interaction can either activate or suppress transcription, depending on the location of target region and the epigenetic status of the promoter[68].

Recent work has characterized the complex that mediates miRNA-dependent transcriptional activation. The core components of this complex are the miRNA-loaded AGO2, the RHA elicase, and CTR9 (a component of PAF1 complex that is involved in transcription initiation and elongation). The complex interacts with the RNA polymerase II thereby stimulating transcriptional initiation and elongation. The recruitment of the activating complex on the promoter is paralleled by specific epigenetic modifications on histones, which are a prerequisite for transcriptional enhancement[69].

Besides controlling transcription, it has been shown that miRNAs can post-transcriptionally mediate the processing of other RNA species in the nucleus. For example, within the nucleus, the metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a target of miR-9 in an AGO2-dependent manner[70]. In a different mechanism, the maturation of miRNAs can be regulated by other miRNAs, such as the maturation of miR-15a/16–1 that is inhibited by miR-709 through binding between pri-miR-15a/16–1 and miR-709[71].

As nuclear miRNAs are involved in the regulation of a variety of oncogenes and tumor suppressors, dysregulation of nuclear miRNA activities is implicated in tumor onset and development[72]. Of note, the ratio between nuclear and cytoplasmic amounts of specific miRNAs has been used as a proxy to distinguish amongst different breast cancer cell lines[73].

RELATIVE EXPRESSION OF ISOMIRS

RNA sequencing conducted in several cancer cell lines and primary tumors has revealed that a miRNA precursor constitutively produces a heterogeneous group of mature miRNAs isoforms showing slightly different lengths[74,75]. These isoforms of a specific miRNAs, named isomiRs, differ from the archetypal sequence on their 5' and/or 3'-end. Paradoxically, in some cases isomiR products are more abundant than the miRBase reference entry.
Importantly, it has been shown that the relative abundance of isomiRs enables the distinction among multiple cancer types[76].

Therefore, for diagnostic purposes, isomir profiling represents an important parameter to associate with global miRNA levels. Unfortunately, though, most detection systems, including commercially available qPCR systems, are not efficient enough to capture single base differences in sequence or termini[77]. NGS is the only system able to reliably detect relative abundance of isomirs in a sample, however to date, this approach is not yet cost-effective for clinical application. Future implementations to detect isomirs are, therefore, needed.

MIRNA DISTRIBUTION BETWEEN FUNCTIONALLY DIFFERENT RISC POOLS

It has been recently reported that mitogenic cues determine the association of mature miRNAs between different isoform of the RISC[78-80]. In particular, miRNAs can be embedded either in high-molecular weight RISC (HMWR) or low-molecular weight RISCs.

Biochemical analyses have shown that the HMWR form contains the core components of the active RISC (AGO and TNRC6 proteins) and it is *bona fide* considered the RISC pool actively engaged in target repression. In contrast, the LMWR form lacks TNRC6 proteins, and therefore it is not enabled to carry out miRNA-mediated repression. As a consequence, the relative abundance of HMWR and LMWR in a given tissue, determines how effectively miRNAs can repress their targets.

For example, the potency by which miR-15/16 family represses a target containing a cognate binding site, increases 40-50% when resting T cells enter exponential proliferation following ex-vivo stimulation. As the expression of miR-15/16 does not increase upon ex-vivo activation, these results may be explained with the fact that activation induces a massive reassembly of the RISC, conceivably reflecting an increased repressory potency of the complex. Resting T cells, indeed, maintain AGO almost completely as part of LMWR, which is completely recruited into HMWR upon activation[78].

These studies for the first time have shown that miRNA expression does not tell us the whole story about the potential of miRNA to control gene expression. In fact, in proliferating cells miRNA abundance may more linearly correlate with miRNA activity than in post-mitotic cells. Therefore, gene expression analysis should take into account differences in RISC activity and should therefore be considered with caution.

These observations indicate that in a given cell, there are two pools of miRNAs: one pool is actively engaged in target repression, and associated with the HMWR, and one pool is apparently excluded from target repression and associated with LMWR. One may hypothesize that the active pool of miRNAs – which conceivably determines the biological properties of the cancer cell– may have a better diagnostic and prognostic value as compared to the whole miRNA pool in the cell, in which also the pool of inactive miRNAs is included.

As this analysis is limited to intracellular miRNAs, it would not apply to circulating miRNAs, which conceivably are not bound to target mRNAs and therefore constitutively associated to a LMWR. However, it may be applied for the characterization of tumors of unknown origin. Another limit inherent to this approach is that fractionation of HMWR and LMWR is required prior sequencing, which makes this approach not practical, given the current fractionation technologies available, and the fact that tumor samples are often treated with fixatives. If this model will be experimentally confirmed in cancer cell lines and primary tumors, it will provide a proof of concept that may stimulate further research in this direction.

CONCLUSIONS

miRNAs have emerged as ideal cancer biomarkers, due to their remarkable stability and their disease-specific expression. Moreover, their presence in serum and plasma at detectable levels makes their profiling extremely accessible by non-invasive approaches. Yet, the information gained by profiling miRNAs' levels has been insufficient to build reliable diagnostic and prognostic workflows in the clinical laboratory. Although more consistent results can be obtained by optimizing sampling procedures, technologies of detection, and data analysis, other characteristics of miRNA, besides global expression levels, can be integrated to gain more detailed information of miRNA function. In particular, we propose that the integration of information regarding subcellular localization, relative abundance of isomirs, editing patterns and distribution between functionally distinct RISCs, may contribute to make miRNAs more powerful cancer biomarkers.

Conflict of Interest:
The Authors declare that they have no conflict of interests.

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