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Molecular profiling and detection methods of microRNA in cancer research

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Abstract: A large portion of human genome was believed to be "useless" and termed as "junk DNA" in the past, given that these sequences did not have any protein coding role. However, with more researchers dwelling into the world of these mysterious genetic codes, a group of non-protein coding RNA (ncRNA) known as microRNAs (miRNAs) is now being recognized to play important roles than they were thought to be. In truth, the first discovery of miRNA was in a simple organism - nematode (scientific name: *Caenorhabditis elegans)*, whereby a mutant displayed aberrant morphological changes. Years after that, researchers then realized that these miRNAs are actually important regulatory molecules — controlling cell division signaling, apoptosis and so on. In fact, the unusual expression of miRNAs has also been associated in etiology of various cancers. Acting like a "double-edge" sword, miRNAs can control and/or act as tumor suppressor genes and oncogenes, thus any unwanted alterations in their expression would bring upon disastrous effects on the host. Therefore, the current review aims to summarize the molecular detection tools that are available for miRNA profiling in cancer research.

Keywords: miRNA; noncoding RNA; detection; profiling; cancer

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Introduction

In the 1972, a geneticist Susumu Ohno coined the concept of "junk DNA", which was used to describe all the non-protein coding regions within the human genome[1]. Since then, scientists around the world have been trying to decode the human genetic code or DNA, particularly to illuminate and understand the genetics of gene regulation and function. Less than twenty years later, the Human Genome Project (HGP) commenced in the early 1990s as an international, collaborative research which is known to be an important milestone in understanding the human genome[2,3]. More importantly, apart from identifying the genes, one of the eight major goals proposed by HGP in 1998 was to elucidate functions of these genes, including non-protein coding sequences. One of the most game-changing discovery was a type of small RNA called microRNAs (miRNAs) in 1993 by Lee and colleagues, in which they cloned the *lin-4* locus almost two decades after its first description and this locus was proven to have exceptional characteristics compared with other normal coding genes[4]. Rather than coding a protein, the *lin-4* gene presents as a small RNA molecule. Two small *lin-4* transcripts with approximately length of 22 and 61 nucleotides were discovered in *Caenorhabditis elegans*; these were significantly smaller in size compared with other genes. Also, *lin-4* mRNA transcripts displayed antisense complementarity to multiple sites in the 3' untranslated region (UTR) of another gene known as *lin-14*, implying that *lin-4* regulates the translation of latter gene via an antisense RNA-RNA interaction. These remarkable breakthroughs subsequently instigated the concept of a unique class of small ncRNA regulatory molecules acting via an antisense-like interaction[5,6]. Accumulating evidence is pointing directly at the role of miRNA in human chronic diseases, including diabetes,

Alzheimer's disease, cardiovascular diseases as well as cancer[7,8]. Carcinogenesis is a very complex, multi-step process which involves at least three stages: initiation, promotion and progression/metastasis[9]. The mutated cells somewhat managed to overcome fate of cell death or apoptosis and subsequently divide and expand itself; some scenarios would bring about life threating events particularly when these mutated cells gain the ability to promote blood vessel formation in order to gain access to nutrients and oxygen supply as well as ability to spread via moving or metastasize to nearby local tissue[9,10]. With the increasing interest in human genome, it was then noted that miRNA expression patterns were certainly tissue-specific, with cancer cells demonstrating significantly different profile than their normal counterpart[11,12]. Furthermore, additional studies also demonstrated that miRNA expression in peripheral blood could be used as an alternative way to "gauge" miRNA expression in the tumor biopsy. Therefore, the current study is to summarize and discuss conventional molecular detection tools as well as some newer, innovative methods that are available currently for miRNA profiling in cancer research.

What are miRNAs and their role in cancer?

miRNAs are recognized as a group of small non-protein coding RNA (ncRNA) sequences with regulatory potential. The biogenesis of miRNA begins with the processing of RNA polymerase II/III transcripts post- or co-transcriptionally, with most of the miRNA being intragenic and processed largely from introns[13]. Two main pathways have been described for miRNA biogenesis: (a) canonical pathway which involves processing by a microprocessor complex an RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) and a ribonuclease III enzyme, Drosha and (b) non-canonical pathway that can be either Drosha/DGCR8-independent and Dicer-independent pathways[13,14]. Nonetheless, it has been shown that animals cannot survive or reproduce normally without miRNAs expression[15]. Using *C. elegans* as an *in vivo* model in genetic study, Sulston and Brenner were able to screen for 300 mutants that gave rise to *C. elegans* displaying diverse developmental defects and behavioral changes in the early 1970s[16]. One of the mutants, designated as lin-4, exhibited elongated and flaccid body morphology with diverse reiterations for particular cell lineages. Little did the scientists noticed that this discovery became the founding member of the miRNA family, whereby it was shown to be involved in the early development of *C. elegans* larval by influencing timing of developmental events across various cell types[17,18]. Nonetheless, the atypical expression of miRNAs has also been associated in etiology of various cancers, given that it can regulate and/or act as tumor suppressor genes and oncogenes[19–21]. For example, Calin and team failed to notice any protein coding genes within chromosome 13q14 and later found that the loss of two miRNAs, miR-15a and miR-16-1 in more than 65 % of B cell chronic lymphocytic leukemia patients[22]. Later on, another study by Bandi and team also revealed that these miR-15a and miR-16 are often deleted or downregulated in non-small cell lung cancer (NSCLC) tissues[23]. Even though the target for these two miRNA seems to be unknown, some studies showed that these miRNA may possess a negative regulatory role for anti-apoptotic B cell lymphoma 2 (Bcl2) protein and eventually induces apoptosis in leukemic cell model[20,24,25]. As such, alterations in miRNA may indeed sound like "total chaos" for the human body, contributing to the hallmark features of human cancer including supporting cell division signaling, dodging growth suppressors, avoiding cell death, activating replicative immortality, increasing invasion and metastasis ability as well as promoting angiogenesis[26–29]. Rather than a loss of function, oncogenic miRNAs or "oncomiR" grant cancer cells survival/growth advantages by evading apoptosis and tend to cleave target mRNAs more frequently than those miRNAs with tumor suppressors function[30–32]. One of the classic example for oncogenic miRNAs would be miR-17-92 which exists as a miRNA polycistron at chromosome 13q31 and is highly expressed in a range of human cancers including lymphoma, lung cancer, breast cancer, colon cancer as well as head and neck cancers[33–38]. All in all, extensive dysregulation of miRNAs can have serious impact on the development of various human cancers, which in turn explains for its importance as diagnostic and prognostic markers as well as targets for new therapeutic agents[39].

Detection and profiling of miRNA

Currently, there are several methods to capture and quantify expression of these small non-coding miRNA. In terms of miRNA detection, there are three main techniques: (a) hybridization-based techniques (e.g. Northern blots, microarrays), (b) amplification-based (e.g. RT-qPCR) and (c) cloning-based (e.g. miRAGE) (Figure 1)[40]. The decision on which techniques to be used for detection is greatly dependent on number of specimens, running time and cost. For examples, Norther blots developed by Alwine and team in the late 1970s remained as the gold standard for gene expression changes and miRNA studies[41]. However, in terms of expression throughput, multiplex RT-PCR and miRAGE can produce results in shorter time, but the newer techniques like microarrays are generally better choices if the study involves large number samples or requires in-depth analysis[40]. Having said that, the quantitative power of these techniques varies; RT-qPCR produces quantitative data, whereas results from Northern blots are semi-quantitative. Hybridization-based technique like *in-situ* hybridization is normally non-quantitative. Each of these techniques have got their advantages as well as limitations, therefore researchers may opt to combine these techniques in a study to improve and/or strengthen their findings.

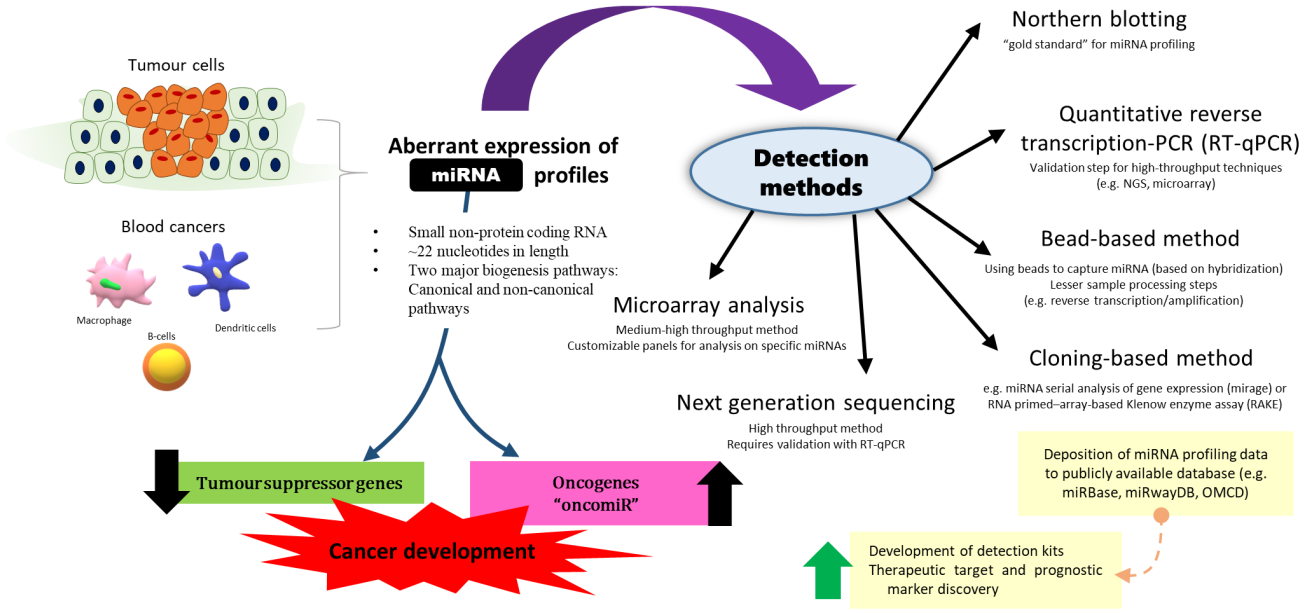

Figure 1. Examples of miRNA profiling detection methods in cancer research.

Northern blotting

Even though close of half a decade has passed since the first introduction of Northern blots, this technique is still the gold standard for miRNA expression profiling. In spite of that, there are several technical considerations which have been raised up by researchers when using Northern blot as a routine miRNA expression profiling tool. As much as the technique stands as a high sensitivity assay, Northern blotting requires huge running time and large amounts (5–25 mg) of total RNA from each sample, on top of potentially dangerous radioactive probes[40].

As a result, researchers have come up with alternatives in probe design, specifically to allow the detection of miRNA without compromising its sensitivity and accuracy. One of which was a technique designed based on the Northern-blot principle by Kim and team, known as LED which consists of three "key players" – digoxigenin-labelled oligonucleotide probes containing locked nucleic acids (LNA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for cross-linkage with membrane[42]. The authors claimed that LED is able to generate clearly visible signals for RNA amounts as low as 0.05 fmol and requires only few seconds of membrane exposure, equivalent to ~1000-fold improvement in exposure-time (Kim et al., 2010). As a non-radioactive label, DIG assay is safer than radioactive methods, while offering the same/similar sensitivity as isotope labeling-based methods[43]. Along with this, locked nucleic acid (LNA)-modified oligonucleotides greatly improve the sensitivity of Northern blotting technique, as high as ten-fold compared to conventional DNA probes[44,45]. LNAs were described as a new class of bicyclic high-affinity RNA analogues, containing a furanose ring in the sugar-phosphate backbone that is chemically locked in an N-type (C3'-*endo*) conformation by the introduction of a 2'-*O*,4'-*C* methylene bridge[45]. Furthermore, Válóczi and team explained that the oligonucleotide probes with LNA was designed in a way that every third nucleotide position was substituted by a LNA monomer. This design subsequently resulted in an increase in its sensitivity yet retained its high specificity, which enables detection of both mature and precursor miRNAs[46]. Similarly, EDC chemically mediates cross linking of RNA to (nylon) membrane which has been shown to improve miRNA detection by up to 25 to 50-fold, depending on plants or mammalian samples[47,48]. Therefore, by combining the goodness of these three components, Kim and team highlighted that LED probes offer multiple advantages; it's more environmentally friendly compared to conventional probes which can be stored for at least six months [42]. Having said that, these "upgrades" seem to incur higher cost compared with the conventional old method, especially when LNA probes can be more expensive than DNA probe and DIG-labeling which will also add up to the final cost.

Microarray-based method

Microarrays are amazing tools in cancer research. Based on nucleic acid hybridizations between target molecules and corresponding complementary probes, it allows genotyping at multiple loci or targets at once. In reality, majority of the published studies reporting miRNA profiling used a variety of microarray technologies. Its high utilization in miRNA profiling studies is fairly self-explanatory, as most of the experimental (and analysis) steps can be done at any molecular biology laboratory and it also allows customization including probes design, chemistry of probe immobilization, labeling of samples and detection of signal[49,50]. At some point, some researchers have also implicated its potential to be used as a standard tool in the near future[51].

With the availability of miRNA databases (e.g. miRBASE, miRwayDB, OMCD), these efforts in turn speeds up the

development of commercially available miRNA microarrays.

In general, a ready-to-use miRNA microarray contains miRNA oligonucleotide probes that have a amine-modified 5'-end which are immobilized onto glass slides by covalent crosslinking[52]. The detection of miRNA type is based on the binding location of fluorescent-dye labelled miRNA on the slides, while its expression level can be quantitated based on the levels of fluorescence emission. Along with this, LNA can also be used in miRNA microarray due to its exceptional affinity and specificity to the complementary RNA. On top of the efficient visualization fostered by the extraordinary thermal stability between LNAs and their target RNA molecules, LNA seems to improve the mismatch discrimination which then lead to multiple attempts to incorporate LNA-based probes in miRNA microarrays design for profiling purposes[53–55]. Exiqon (now under Qiagen) used to manufacture microarray platform with probes containing LNA bases, providing higher annealing affinities[49]. As a result, several studies attempted to compare the performance of commercial miRNA expression array platforms[49,56]. Git and team included six commercially miRNA expression array platforms which consist of both single- and dual-channel fluorescence technologies in their studies[49]. Based on their studies, all six platforms tested were equally applicable to cell line and tissue, but factors such as input sample amount may need to be considered when selecting a platform to be used. On the other side, another study compared four platforms including Agilent, Illumina (platform withdrawn since 2010), Exiqon and Miltenyi[56]. It was noted that higher modulated miRNAs were identified via Agilent and Illumina platforms for class comparison analysis between tumor and normal samples. Even though Exiqon did not produce the same number of modulated miRNAs as the two platforms, most of miRNAs modulated in Agilent and Illumina were detectable, although they did not reach statistical significance. On the contrary, the same miRNAs were mostly undetected on the Miltenyi platform which can be explained by its sensitivity to GC content. Sah and colleagues found similar results with Ambion and Agilent which exhibite better accuracy while Illumina and Exiqon displayed higher specificity[51]. Another company, Invitrogen also developed a series of microarray for miRNA profiling, known as NCode™ Multi-Species miRNA Microarray. Tchernitsa and team used the Invitrogen NCode(™) Multi-Species miRNA Microarray Probe Set containing 857 mammalian probes to study six primary gastric cancers (compared with normal/ non-cancerous tissue); out of which three of them presented lymph node metastases, while the other three did not[57]. Comparing gastric carcinoma with non-cancerous tissue, twenty miRNAs were differentially regulated and six of these miRNAs showed distinct expression which separated node-positive from node-negative gastric cancers, including miR-103, miR-21, miR-145, miR-106b, miR-146a, and miR-148a. These results indeed emphasized the importance of microarray as high throughput method in miRNA studies. Despite of that, researchers have also pointed out that the periodic changes to miRNA database like miRBase then imposes a reannotation of microarray and qPCR probes prior to analysis. Nevertheless, this would also provide researchers the flexibility in selecting types of microarrays to be used, be it pre-designed microarrays or customized microarrays.

PCR-based technique: reverse transcription quantitative PCR (RT-qPCR)

Even though there are several options which allow measurement of multiple target miRNAs simultaneously, RT-qPCR remains one of the popular choice for miRNA profiling as it's an important technique for validating expression data obtained from high-throughput screening (e.g. with microarray).The first step of RT-qPCR begins from precise and absolute conversion of RNA into complementary DNA (cDNA) via reverse transcription. However, given its nature of miRNA which has a limited length (~22 nucleotides), reverse transcription can be difficult to perform. Apart from that, mature miRNA sequence can exist in two forms – pre- and the pri-miRNAs, along with little or no common sequence feature to be used for their enrichment and amplification[58]. Before quantification, there are two approaches for reverse transcription of miRNAs: (a) by using miRNAs-specific reverse transcription primers or (b) using a universal primer that targets the miRNAs that are tailed with a common sequence (e.g. poly-A tail). While the use of miRNA-specific primers (MSPs) reduces background "noise", using a universal reverse transcription method is useful when there are several different miRNAs needed to be studied from a small amount of input. Additionally, there is another alternative which enables multiple miRNAs to be reverse transcribed by pooling stem-loop primers[59,60]. Step-loop primers consist of a short single stranded sequence at their 3'-ends that anneals to the 3'-end of the miRNA of interest, a double-stranded segment (the stem) and a loop. Due to its structure, the primer will not be able to bind to pri- and pre-miRNAs and to any dsDNA that may be present.

RT-qPCR possesses numerous benefits compared to microarray as it presents higher speed and sensitivity as well as larger dynamic range[40,61]. Furthermore, RT-qPCR also requires low amounts of starting specimen which makes it more user-friendly. At the time of writing, there is currently over 1,900 human miRNA curated in miRBase (http://www.mirbase.org/). As single target RT-qPCR can be both time and reagent consuming, several strategies can be used to perform parallel reverse transcription when there is a need to detect a large number of miRNAs in a single sample. A study by Tang and colleagues incorporated 220 individual stem-loop primers in their study to develop a multiplex reverse transcription assay[62]. A pre-PCR process was included to reduce significant loss of detection sensitivity using low amounts of RT-qPCR primers (pre-PCR) and subsequently the cDNA product was diluted before multi-well plates containing MSPs, universal primers and TaqMan probes for RT-qPCR.

Apart from that, there are also miRNA arrays available for

the use of miRNA profiling. Zhang and team measured differential expression of 95 miRNAs in pancreatic cancer tissues and cell lines by RT-qPCR using the QuantiMir System (SBI System Biosciences)[63]. As a consequence, unique miRNA profiles were seen in pancreatic cancer tissues and cell lines which reflected individual diversity, compared to adjacent normal pancreatic tissue or cells. After another validation using RT-qPCR, a total of eight miRNA were upregulated in most of pancreatic cancer tissues and cell types. On the other hand, as a medium-throughput method for RT-qPCR, Taqman array microRNA cards (by Applied Biosystems) combine microfluidics technique and classic/advance chemistries which can measure up to 384 miRNAs. Based on project's needs and directions, user can also select to use either pre-designed (standard) or custom-made cards. Mees and colleagues used TaqMan Low density microRNA Arrays (TLDA) to investigate miRNA expression in ductal adenocarcinomas of the pancreas[64]. The main advantage is that this system allows as little as 1 ng of RNA in circumstances whereby pre-amplification PCR step is performed[65]. For this approach, each TLDA card contains pre-loaded primers and TaqMan probes to amplify a single miRNA[64,65]. After cDNA synthesis using predefined pools of reverse transcription primers, cDNAs are loaded into the micro fluidic card where amplification of individual miRNA occurs[64]. This method provides a simple and convenient way for parallel monitoring of a large number of miRNAs by using RT-qPCR.

Conversely, there are several innovative methods for quantification of miRNA such as LNA-enhanced primers with droplet digital PCR (ddPCR) and the Two-tailed RT-aPCR method which uses primers composed of two hemiprobes (and connected by a hairpin structure)[66-69]. The inclusion of LNA in the forward PCR primers keeps its sequence to be short but highly specific and detects most of the miRNA sequences, while in the reverse primer binds to the 3′ end of the miRNA sequences[66]. Andreasen and team concluded that this unique combination confers specificity plus extreme sensitivity thus making it useful especially for quantification of miRNAs in difficult samples like FFPE tissues. Complementing to the strategy of using LNA-enhanced primer, a relatively new technology that serves the same purpose as qPCR machines comes into the picture, known as ddPCR[69]. This technique eludes several problems with conventional qPCR such as the need for reference gene or replicate samples as the system detects fluorescence signals in the nanoliter-sized water-in-oil droplets containing target molecule[70,71]. Using FFPE specimens, Laprovitera and team used a customized, pre-spotted 96-well plates to study the expression of 92 miRNA with different miRCURY LNA miRNA primers (Qiagen, former Exiqon)[69]. The customized plate was designed to cover most cancer-specific miRNAs (including 89 cancer-specific miRNA and 3 reference genes). In their study, the team managed to detect miRNAs expression in 14 FFPE specimens representing different type of tumors comprising of liver, skin, breast, gastric, colon, ovary, prostate, gastrointestinal-neuroendocrine and so on. Even though there wasn't a direct comparison between this technique and microarray in the same study, the team observed a highly significant correlation ($p < 0.0001$, Spearman $r > 0.7$) between results generated from this technique and their previous microarray experiment (after data normalization).

In 2017, Androvic and team introduced a novel and cost-effective strategy to quantify miRNA expression known as the Two-tailed RT-qPCR[67]. As the primers are made up of two hemiprobes (which are complementary to different regions of target miRNA) with a oligonucleotide tether folded into a hairpin structure, this design then subsequently increase the binding strength to template, increasing its sensitivity while detecting all terminal variants of any miRNA (isomiRs). In another words, this assay enables a "true" reflection or measurement of total miRNA content (of interests) in a sample. Having the advantage of allowing multiplex during the reverse transcription step, the miRNA profiles generated by Two-tailed RT-qPCR displayed excellent correlation with the standard TaqMan miRNA assays ($r^2 = 0.985$). Following the success of developing the rapid technique for miRNA expression with a total analysis time of less than 2.5 hours, the team led by Androvic subsequently published another work last year, highlighting the use of this technique as a quality control test for circulating miRNA studies[68]. As a matter of fact, even though RT-qPCR is described as a "gold standard" for typical gene expression studies, its application in miRNA profiling may be limited by the constantly increase number of miRNA on a genomic scale, thus suggesting its utilization as a validation test for other high-throughput techniques such as microarray and next generation sequencing (NGS) which might be a more affordable plan (depending on factors such specimen and miRNA target numbers).

Next generation sequencing (NGS)

In biomedical science, Sanger sequencing has always been the principal approach and gold standard for DNA sequencing[40]. Two decades ago, a team from Lynx Therapeutics (USA) (which was later acquired by Illumina) launched the first NGS technologies known as Massively Parallel Signature Sequencing (MPSS)[72]. The technological feature of this method is that clonally amplified or single DNA molecules in MPSS are spatially separated in a flow cell, making it different from the Sanger sequencing which works based on the electrophoretic separation of chain-termination products produced in individual sequencing reactions[40]. As a general rule, NGS technologies involve repeated cycles of nucleotide extensions mediated by polymerase or by iterative cycles of oligonucleotide ligation in one format[73]. NGS platforms available today are Roche 454 GS FLX sequencing, Illumina/Solexa Genome Analyzer sequencing, Applied Biosystems/SOLiD as well as Helicos Biosciences and Single-Molecule, Real-Time (SMRT) Sequencing by Pacific Biosciences (PacBio).

In truth, quite a number of known miRNAs were discovered by conventional cloning and Sanger sequencing approach[74]. However, the sequencing power of NGS opens up a new window for researchers to discover novel miRNAs as it is not hindered by variability in melting temperatures, neither co-expression of almost identical miRNA family members nor post-transcriptional modifications as in other molecular techniques like microarray and RT-qPCR. At earlier times, several groups have attempted to use different NGS

technologies to uncover novel miRNA or even generate miRNAome. In 2010, Ramsingh and team used 454-based sequencing to study miRNAome in a patient suffering from acute myeloid leukemia[75]. A total of 472 miRNA (including 7 of them being novel) was identified from leukemic myeloblasts; some of them showed differential expression compared to normal (healthy) $CD34^+$ cells. Besides that, Creighton and team took advantage of another platform — Illumina/Solexa Genome Analyzer sequencing to discover of novel miRNAs in female reproductive tract[76]. In the same study, they have added nearly 100 putative novel miRNA (with mid-high confidence) derived from various organs of the female reproductive system (in both diseased and normal states), representing diseases such as ovarian cancer, endometriosis, and uterine tumors (benign and malignant). Comparing two different systems, the Illumina platform (e.g. HiSeq) which uses typically generates more reads at lower cost compared to Roche 454 pyrosequencing system[53,77]. Furthermore, one of the main problems with the latter system is that it has got relatively high error rate (for poly-bases longer than 6 bp), even though the system does offer an automated process for library construction. Even so, Applied Biosystems purchased SOLiD or Sequencing by Oligo Ligation Detection sequencing in 2006, which stands as a two-base sequencing technology based on ligation. Schulte and colleagues have adopted SOLiD NGS in their study analysing small RNA transcriptomes of neuroblastoma cases. The team subsequently successfully revealed the differential expression of miRNAs in favourable versus unfavourable neuroblastoma[78].

The third generation of NGS was firstly introduced in 2008 and defined as single molecule sequencing which was entirely different from the clonal based second-generation sequencing methods[79,80]. The short running time of SMRT sequencing by PacBio makes it particularly attractive for diagnostic use. A research team in India performed IsoSeq analysis on infratentorial ependymoma tumor tissue using SMRT technology, PacBio RSII[81]. A total of 2952 unique transcripts were identified to be involved in 307 KEGG pathways, with 22 transcripts coding 18 genes related with miRNA biosynthesis/processes (KO0520622). With the fast development in NGS technology, the fourth generation of NGS technology such as the MinION, a commercially available device from Oxford Nanopore Technologies (ONT) has also begun to gain attention as a potential tool for miRNA profiling work[82,83]. Even though it comes at a lower pricing compared to the third generation NGS, it is rather unfortunate to mention that more work/modification may need to be done to overcome the compatibility issue of the technology for short nucleic acid strands or miRNA due to their short length[83,84].

As much as NGS offers as somewhat powerful tool to identify and detect miRNA in various samples, some researchers pointed out that there are possibilities of bias resulting from RNA ligation and amplification steps[49,85]. Furthermore, the increasing amount of data generated from these NGS technologies require high computation power for analysis. Also, for high-throughput methods like NGS, additional data validation step is still required using other techniques like qRT-PCR[86,87].

Bead-based method

Apart from techniques mentioned above, some researchers developed another exciting method for the profiling of miRNA expression which works based on "beads" that capture different types of miRNA. Luminex Corporation developed the bead-based array known as xMAP™ system, which is a multiplexed microsphere-based suspension array (http://www.luminexcorp.com/technology/index.html). For this method, the oligonucleotide-capturing probes complementary to miRNAs of interest are linked to carboxilated 5-micron polystirene beads impregnated with mixture of two fluorescent dyes, each coded for a single miRNA[88]. In 2005, Lu and colleagues were able to differentiate tumors that were inaccurately classified by mRNA profiles[89], while another study in United Kingdom discovered new markers of human breast cancer subtype[90]. The bead-based miRNA arrays offers several benefits compared to glass-slide microarrays: (a) user-friendly and easy to use, (b) relatively low cost with advanced statistical performance, (c) faster hybridization kinetics and (d) higher flexibility in preparation of the array[40]. This technique remains as a popular tool in cancer research studying miRNA expression at present day. Wang and team studied the expression of miRNA in NSCLC tissues using Luminex xMAP bead-based suspension array and highlighted that the system requires as little as 2 μL of sample volume without the need of running reverse transcription or amplification step[91]. Though, this method demands for specialized equipment, thus limiting its usage.

Others: Cloning based assay, miRAGE and RNA primed–array-based Klenow enzyme assay (RAKE)

Before witnessing the major breakthrough in developing NGS techniques, researchers have heavily relied on traditional methods like amplification and cloning methods to increase the efficiency of small RNA species discovery including miRNA. miRNA serial analysis of gene expression or miRAGE was developed by Cummins and colleagues that merged the aspects of direct miRNA cloning and SAGE[92,93]. While allowing identification of new miRNA, miRAGE is similar with conventional cloning approaches: it starts with the isolation of 18-26 base RNA molecules and then ligation with specialized linkers and adapters before reverse-transcription step into cDNA[93,94]. The cDNA then will be subjected to another PCR reaction with the help of biotinylated primers before purification step with column of streptavidin-coated beads (to remove biotin tagged linkers). The eluded product will be purified miRNAs and these miRNAs are then concatenated, cloned, and sequenced for analysis. The main advantage of this approach is that it is able to generate large concatemers, enabling as many as 35 tags to be identified in a single sequencing reaction, whereas existing cloning protocols analyse approximately five miRNAs per reaction[92].

Aside from that, there is another method available for miRNA detection known as RNA-primed, array-based Klenow enzyme (RAKE) assay developed by Nelson and colleagues in 2004[95]. As it doesn't require any sample RNA manipulation prior to hybridization, the un-modified miRNA is hybridized to immobilized DNA probes. Biotinylated dATP is incorporated to DNA probe with hybridized miRNAs acting as primers by Klenow enzyme and subsequently a streptavidin-conjugated fluorophore is applied to visualize and analyze the expression of miRNAs. RAKE offers a high throughput protocol with unique advantages for specificity over northern blots or other microarray-based expression profiling platforms[96]. Moreover, the team demonstrated that miRNAs can be isolated and profiled from FFPE tissue, which provides another alternative for analyses of small RNAs from archival human tissue. On top of that, Berezikov and colleagues proved that novel mammalian miRNA candidates can be identified by extensive cloning and RAKE analysis[97].

Conclusion and future recommendations

The continuous expansion of miRNA knowledge has deemed the importance of these small non-coding nucleic acid in the area of cancer research, particularly as biomarkers in diagnostic and therapeutic targets in drug discovery field[98]. Even though newer methods are constantly being developed/introduced for miRNA profiling use, there is still much room for improvement, given that these tools are still unable to replace the conventional method that "sits on the throne". One of the bottlenecks would be experimental confirmation of miRNA targets behind these phenotypes computed via data generated from high-throughput technology. Upon obtaining a comprehensive understanding on their roles, selected miRNA could be used in RNA interference strategy to achieve therapeutic effects against different types of human cancers[99]. In fact, a quick search on clinical trials registry website (e.g. clinicaltrial.gov) revealed that there is a total of 327 studies related to miRNA, out of which 98 studies were interventional studies (as of 8th June 2020). However, no completed results reported results related to the use of miRNA as therapeutic agent(s) on the database yet. Just like any other drugs, it is impossible to observe success with every miRNA tested, but the "magic bullet" would pledge a promising future in the war against cancer.

Authors contribution

The literature review and manuscript writing were performed by NSAM and H-LS. NSAM and II provided vital guidance of the research and proof of the writing.

Conflict of interest

The authors declare that there is no conflict of interest in this work.

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