Clinics of Surgery

Research Article ISSN 2638-1451 |Volume 6

Importance of Micrornas in Human Cancer Development: A Molecular Analytical Approach

Ahmed FE[1*], Gouda MM[2,3] and Ahmed NC[4]

[1,4]GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Drive, Greenville, NC 27834, USA

[2,3]Egypt National Research Center, Giza, Dokki, Cairo, Egypt, and College of Biosystems Engineering and Food Science, Zhejang University, 866 Yuhangtang, Hangzhou, 310058, China

[*]Corresponding author:

Farid E. Ahmed,
GEM Tox Labs, Institute for Research in Biotechnology, 2905 South Memorial Drive, Greenville, NC 27834, USA.
E-mail: gemtoxconsultants@yahoo.com

Received: 12 Aug 2021
Accepted: 24 Aug 2021
Published: 30 Aug 2021

Citation:

Ahmed FE. Importance of Micrornas in Human Cancer Development: A Molecular Analytical Approach. Clin Surg. 2021; 6(4): 1-30

1. Abstract

Common steps in analysis of microRNA expression levels between different tissues, developmental stages, or disease states is to study microRNA expression levels by several methods as: NGS, microarray analysis, real-time PCR, Northern blots, in situ hybridization, and solution hybridization. Of these techniques, quantitative reverse transcription Polymerase Chain Reaction (qRT-PCR) is one the most sensitive and accurate method. For qRT-PCR applications, the tools include: a) Effective method of microRNA isolation from samples; b) RT-qPCR reagents optimized for microRNA detection; c) Assays specific to the microRNAs of interest, and d) Real-time analytical instruments and reagents validated for microRNA detection. MicroRNAs have also been employed diagnostically, using liquid biopsies.

Growing interest and utility of Circulating Cell-Free DNA [cfDNA] and interest in their role in oncology re-search is continue to grow in importance, in order to exploit their role as biomarkers for detecting premalignant and early stage cancers. The field of microRNA-based cancer research has witnessed a remarkable evolution over the last two decades, is the role of microRNAs as disease prognostic biomarkers, as well as recent attempts to exploit their role as therapeutic targets, as their small size and their stability in a variety of body fluids make them attractive substrates for employment as biomarkers. Current approaches for detecting microRNAs in blood and other body fluids is inadequate. The advantage of using microRNA approach is based on concurrently tar-geting multiple effectors of pathways involved in cell differentiation, proliferation, as well as in cell survival.

In this review, we have employed regulatory small microRNAs as unifying molecules, which have shown a strong correlation with induction and progression of many human cancers, as they progress from the non- to the invasive stages of various types of human cancers, as detailed in this review below.

2. Main Common Human Cancers in The USA

Cancer is a group of diseases involving abnormal cell growth with the potential to invade other body parts, in contrast with benign tumors that do not spread. Possible signs and symptoms include a lump, abnormal bleed-ing, prolonged cough, unexplained weight loss and a change in bowel movement. While these symptoms may indicate cancer, they are also other causes. Over 100 types of cancers affect humans. The 13 most common can-cers in the USA (out of ~ 200) [1], represent approximately 71.5% of all estimated yearly new cases, for year 2018 are breast cancer, lung cancer, prostate cancer, colorectal cancer, melanoma skin cancer, bladder cancer, non-Hodgkin's lymphoma, kidney cancer, endometrial cancer, leukemia, pancreatic cancer, thyroid cancer, and liver & intrahepatic bile duct cancer [2, 3], as tabulated in (Table 1).

Table 1: Estimated Yearly New Cases of the 13 Most Common Cancers in the USA

Cancer Type	Estimated New Cases			Estimated Yeearly Deaths			Five years Survival rates		
	Female	Both Sexes	Male	Female	Both Sexes	Male	Female	Both Sexes	Male
Breast	268,600		2,670	41,760		500	90%		
Lung		228,150§						23%§	
Prostate		164,690		142,670				98%	
Colorectal		145,600§			51,020§			64%§	
Skin Melanoma		96,480§			7,230§			92%§	
Bladder Cancer		80,470§			17,670§			77%§	
Non-Hodgkin's Lymphoma		74,200§			19,970§			71%§	
Kidney		73,820§			14,770§			75%§	
Leukemia (all types)		61,880§			12,160§			84%§	
Pancreatic		61,780§			22,840§			61.4%§	
Thyroid**		56,770§			45,750§			9%§	
Liver***		2,070§			2,170§			~100%§	
	41,030§		31,780§						18%§

Table 1: Methods to isolate Exosomes

Isolation method	Mechanism	Advantages	Disadvantages
Differential centrifugation	The method consists of several centrifugations aiming to remove cells, large vesicles and debris and precipitate exosomes.	Differential centrifugation is the standard common method used to isolate exosomes from biological fluids and media.	The efficiency of the method is lower when viscous biological fluids such as plasma and serum are used for analysis.
Density gradient centrifugation	This method combines ultracentrifugation with a sucrose, or iodixanol, density gradient.	The method allows separation of the low-density exosomes from other vesicles, particles and contaminants.	Very high sensitivity to the centrifugation time.
Size exclusion chromatography	Size-exclusion chromatography separates macromolecules on the base of their size. It applies a column packed with porous polymeric beads.	The method allows the precise separation of large and small molecules and application of various solutions. Compared to centrifugation methods, the structure of exosomes isolated by chromatography is not affected by shearing force.	The method requires a long running time, which limits applications of chromatographical isolation for processing multiple biological samples.
Filtration	Ultrafiltration membranes are used to separate exosomes from proteins and other macromolecules. The exosomal population is concentrated on the membrane.	Filtration allows separation of small particles and soluble molecules from exosomes. During the process the exosomal population is concentrated by the filtration membrane.	Exosomes can adhere to the filtration membranes and become lost for the following analysis. Also, since the additional force is applied to pass the analyzed liquid through the membranes, the exosomes can potentially be deformed or damaged.
Polymer-based precipitation	The technique includes mixing the biological fluid with polymer-containing precipitation solution, incubation step and centrifugation at low speed.	The advantages of precipitation include the mild effect on isolated exosomes and usage of neutral pH.	Polymer-based precipitation methods co-isolate non-vesicular contaminants, including lipoproteins. Also, the presence of the polymer material may not be compatible with down- stream analysis
Immunological separation	Various immunological methods are applied. Magnetic beads bound to the specific antibodies are used to isolate exosomes. Also, ELISA-based separation method was developed.	The method allows isolation of all exosomes or selective subtypes of exosomes. Also, it may be applied for characterization and quantification of exosomal proteins.	The method is not applicable for large sample volumes. Also, the isolated vesicles may lose the functional activity.
Isolation by sieving	This technique isolates exosomes by sieving them via a membrane and performing filtration by pressure or electrophoresis.	Relatively short separation time and gives high purity of isolated exosomes.	Low recovery of isolated exosomes.

3. Cancer Types

Cancers are often described by the body part that they originated in. However, some body parts contain multiple types of tissue, so for greater precision, cancers are additionally classified by the type of cells that the tu-mor cells have originated from. These various cancer types include:

3.1. Carcinoma: These are cancers derived from epithelial cells, starting in the skin or tissue that line other organs and tissues throughout the body, and they include many of the most common cancers, particularly in older adults. Nearly all cancers developing in the breast, prostate, lung, pancreas and colon are carcinomas.

3.2. Sarcoma: Is cancer arising from connective tissues, such as bones, muscles, cartilage, fat, nerve & blood vessels.

3.3. Lymphoma and Leukemia: These two classes of cancer arise from cells that make blood. Lymphoma is cancer of the lymph gland, whereas leukemia is cancer of the bone marrow, which creates blood cells. Melanoma: Is cancer of the skin, which starts in the pigment melanin. Lymphoma and melanoma are cancers of the immune system.

3.4. Germ Cell Tumor: These are cancers that are derived from pluripotent cells, most often presenting in the testi-cle or the ovary, thus they are referred to as seminoma and dysgerminoma, respectively. Blastoma: Cancers derived from immature "precursor" cells or embryonic tissue. Blastomas are more common Cancers are usually named using -carcinoma, -sarcoma, -lymphoma, -melanoma or -blastoma as a suffix, with the Latin or Greek word for the organ or tissue of origin as the root [2-6]. Tobacco use is the cause of about 22% of cancer deaths [3]. Another 10% are due to obesity, poor diet, lack of physical activity, or excessive drinking of alcohol [5-13]. Other factors include certain infections, exposure to ionizing radiation and environmental pollu-tants [3]. In developing countries, 15% of cancers are due to infections such as Helicobacter pylori, hepatitis B, hepatitis C, human papilloma virus infection, Epstein–Barr virus and human immunodeficiency virus (HIV) [3]. These factors act, at least partly, by changing the genes of a cell. Typically, many genetic changes are required before cancer develops [14]. Approximately 5–10% of cancers are due to inherited genetic defects from a per-son's parents [15]. Cancer can be detected by certain signs and symptoms or screening tests. It is then further investigated by medical imaging, and is confirmed by using of biopsy [16]. Many cancers can be prevented by not smoking, maintaining a healthy weight, not drinking too much alcohol, eating plenty of vegetables, fruits, whole grains and vaccination against certain infectious diseases, not eating too much processed and read meat, and avoiding too much sunlight exposure [17]. Early detection through screening is useful for cervical and colo-rectal cancer. The benefits of screening for breast cancer are controversial [18-20]. Cancer is often treated with some combination of radiation therapy, surgery, chemotherapy and targeted therapy [5]. Pain and symptom management are an important part of care. Palliative care is particularly important in people with advanced dis-ease [2]. The chance of survival depends on the type of cancer and extent of disease at the start of treatment. In children under 15 at diagnosis, the five-year survival rate in the developed world is on average 80%. For cancer in the United States, the average five-year survival rate is 66% [8].

In 2015, about 90.5 million people had cancer [9]. About 14.1 million new cases occur a year (not including skin cancer other than melanoma) [22] It caused about 8.8 million deaths (15.7% of deaths). The most common types of cancer in males are lung cancer, prostate cancer, colorectal cancer and stomach cancer [19]. In females, the most common types are breast cancer, colorectal cancer, lung cancer and cervical cancer [20]. If skin cancer other than melanoma were included in total new cancer cases each year, it would account for around 40% of cases [21, 22]. In children, acute lymphoblastic leukemia and brain tumors are most common, except in Africa where non-Hodgkin lymphoma occurs more often. In 2012, about 165,000 children under 15 years of age were diagnosed with cancer [23]. The risk of cancer increases significantly with age, and many cancers occur more commonly in developed countries. Rates are increasing as more people live to an old age, and as life style changes occur in the developing world. The financial costs of cancer were estimated at $1.16 trillion US per year as of 2010 [22, 24]. All tumor cells show the six hallmarks of cancer. These characteristics are required to pro-duce a malignant tumor. They include: a) cell growth and division absent the proper signals, b) continuous growth and division even given contrary signals, c) avoidance of programmed cell death, d) limitless number of cell division, e) promoting blood vessel formation, e) programmed cell death, and f) invasion of tissue and for-mation of metastases [25]. The progression from normal cells to cells that can form a detectable mass to outright cancer involves multiple steps known as malignant progression [26].

4. History of Cancer

4.1. Early Era

The earliest known descriptions of cancer appear in Ancient Egyptian paperi. The Edwin Smith Papyrus was written around 1600 BC (possibly a fragmentary copy of a text from 2500 BC) contains description of cancer, as well as a procedure to remove breast tumors by cauterization [27]. Hippocrates (ca. 460 BC – ca. 370 BC) described several kinds of cancer, referring to them as karkinos (carcinos), the Greek word for crab or crayfish, as well as carcinoma. This name comes from the appearance of the cut surface of a solid malignant tumor, with the veins stretched on all sides, as the crab has its feet, hence it derives its name [28]. Since it was against Greek tradition to open the body, Hippocrates only described and made drawings of outwardly visible tumors on the skin, nose, and breasts. Treatment was based on the humor theory of four bodily

fluids (black and yellow bile, blood, and phlegm). According to the patient's humor, treatment consisted of diet, blood-letting, and/or laxa-tives. Celsus (ca. 25 BC - 50 AD) translated karkinos into cancer, the Latin word for crab or crayfish. In the 2nd century AD, the Greek physician Galen used oncos (Greek for swelling) to describe all tumors, reserving Hip-pocrates' term carcinos for malignant tumors. Galen also used the suffix -oma to indicate cancerous lesions. It is from Galen's usage that we derive the modern word oncology [29]. Through the centuries, it was discovered that cancer could occur anywhere in the body, but Hippocrates' humor-theory based treatment remained popular until the 19th century with the discovery of cells [30].

4.2. Modern Era

In the 16th and 17th centuries, it became more acceptable for doctors to dissect bodies to find out the cause of death. The German professor Wilhelm Fabry believed that breast cancer was caused by a milk clot in a mam-mary duct. The Dutch professor Francois de la Boe Sylvius, a follower of Descartes, believed that all disease was the outcome of chemical processes, and that acidic lymphatic fluid was the cause of cancer. His contempo-rary Nicolaes Tulp thought that cancer was a poison that slowly spreads, and that it was contagious [31]. The first cause of cancer was identified by British surgeon Percival Pott in 1775, after he found out that cancer of the scrotum was a common disease among chimney sweeps. With the use of the microscope in the 18th centu-ry, it was found out that the 'cancer poison' eventually spreads from the primary tumor through the lymph nodes to other sites (metastasis), a view that was first formulated by the English surgeon Campbell De Morgan between the year of 1871 to 1874 [32]. The use of surgery to treat cancer led to poor results because of poor hygienic practices. In the 19th century, asepsis improved surgical hygiene and as the survival statistics improved, surgical removal of the tumor became the primary treatment for cancer. In the late 19th century, the idea that the body was made up of various tissues, which in turn were made up of millions of cells, laid rest the humor-theories about chemical imbalances in the body [27].

5. Epidemiology

Estimates are that in 2018, 18.1 million new cases of cancer and 9.6 million deaths occur globally. About 20% of males and 17% of females will get cancer at some point in time while 13% of males and 9% of females will die from it [33]. In 2008, approximately 12.7 million cancers were diagnosed (excluding non- melanoma skin cancers and other non-invasive cancers), and in 2010 nearly 7.98 million people died [34]. Cancers account for approximately 16% of deaths. As of 2018, the most common cancers are lung cancer (1.76 million deaths), colorectal cancer (860,000), stomach cancer (780,000), liver cancer (780,000), and breast cancer (620,000), mak-ing invasive cancer the leading cause of death, the second leading cause, and over half of cases occurring in the developed countries [2, 3]. Deaths from cancer were 5.8 million in 1990. Deaths have been increasing primarily due to longer lifespans and lifestyle changes in the developing world [34]. The most significant risk factor for developing cancer is age [35]. Although it is possible for cancer to strike at any age, most patients with invasive cancer have been observed (35). It is believed that if one lives long enough, sooner or later he/she will get cancer [25]. Some of the association between aging and cancer is attributed to immunosenescence [36], as errors accu-mulate in DNA over a lifetime [37], and age-related changes occur in the endocrine system [38]. Aging's effect on cancer is complicated by factors such as DNA damage and inflammation promoting, whereas factors such as vascular aging and endocrine changes inhibiting it [39].

Some slow-growing cancers are particularly common, but often are not fatal. Autopsy studies in Europe and Asia showed that up to 36% of people have undiagnosed and apparently harmless thyroid cancer at the time of their deaths, and that 80% of men develop prostate cancer by age 80 [40, 41]. As these cancers do not usually cause patients' death, identifying them would have represent over diagnosis, rather than useful medical care [3]. The three most common childhood cancers are leukemia (34%), brain tumors (23%) and lymphomas (12%) (42). In the USA, cancer affects about 1 in 285 children [43]. Rates of childhood cancer increased by 0.6% per year between 1975 and 2002 in the USA (44), and by 1.1% per year between 1978 and 1997 in Europe [42]. Death from childhood cancer decreased by half between 1975 and 2010 in the USA [43].

6. Causes of Cancer

Cancer is a disease caused by genetic changes leading to an uncontrolled cell growth and tumor formation. The basic cause of sporadic (non-familial) cancers is DNA damage and genomic instability, leading to mutations that inactivates suppressor genes, which causes cancer to development [45]. See (Figure 1). A minority of cancers are due to inherited genetic mutations [46]. Most cancers are related to environmental, lifestyle, or behavioral exposures [47]. Cancer is generally not contagious humans, though it can be caused by oncoviruses and cancer bacteria [47] The term "environmental" refers to everything outside the body that interacts with humans [48]. The environment is not limited to the biophysical environment (e.g. exposure to factors such as air pollution or sunlight), but also includes lifestyle and behavioral factors [49].

Over one third of cancer deaths worldwide (and about 75–80% in the USA) are potentially avoidable by re-ducing exposure to known factors [50, 51]. Common environmental factors that contribute to cancer death in-clude: a) exposure to different chemical and physical agents (tobacco use accounts for 25–30% of cancer deaths), b) environmental pollution c) diet and obesity (30–35%) d) infections (15–20%) e) radiation (both ionizing and non-ionizing, up to 10%) [52]. These factors act, at least partly, by altering the function of genes within cells; typically, many such genetic

changes are required before cancer develops [53]. Aging has consistently been regarded as an important factor when evaluating the risk factors for the development of par-ticular cancers. Many molecular and cellular changes involved in the development of cancer accumulate during the aging process and eventually manifest as cancer [54].

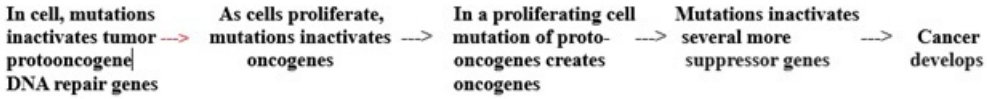

Figure 1: Cancer requires multiple mutations that inactivate DNA repair, proto-oncogenes and suppressor genes, enhanced during aging, to progress into full blown cancer

Isolate Total RNAs → Reverse transcribe total small RNA into cDNA → Carry out NGS/ Microarray studies → Analyze NGS & microarray data → Chose a smaller number of samples to run RT-qPCR, or Absolute PCR reactions → Analyze quantitative PCR data

Figure 1: MicroRNA Quantification Experimental Workflow for a relative qPCR, or for Absolute PCR.

7. Symptoms

Symptoms are changes in the body caused by the presence of cancer, caused by the effect of cancer on the part of the body where it is growing, although the disease can also cause more general symptoms such as weight loss or tiredness. There are more than 100 different types of cancer with a wide range of different signs and symptoms which can manifest in different ways. Typical symptoms of cancer include: the presence of unusual lump in the body; changes in a mole on the skin, such as size, color or shape thickness; a persistent cough or hoarseness; change in bowel habits, such as unusual movement; difficulty in swallowing or continuity; ab-normal bleeding, including bleeding from the vagina, or blood in urine or feces; a persistent sore or ulcer; Diffi-culty passing urine; sudden weight loss, pain or tiredness/fatigue, or night sweats; pains (headaches), as well as abdominal pain; and skin changes such as a rash or an unusual texture [55, 56].

7.1. Increased Lactate Production

The Warburg effect attests that cancer cells in the presence of oxygen and glucose take a different path of energy production, as cancer cells convert glucose in the presence of oxygen into lactate through lactate dehy-drogenase, instead of employing pyruvate though the Citric Acid Cycle (CAC), also known as the Tricarboxylic Acid Cycle (TCA) or Krebs cycle used by aerobic organisms to release stored energy through the oxidation of acetyl-coA-derived from carbohydrates, fats and proteins into Adenosine Triphosphate (ATP) and Co2 (oxidative phosphorylation) [57]. Cancer cells can still carry out oxidative phosphorylation, not primarily for the purpose of energy production, but for biomass production through utilizing the intermediates from TCA cycle. This unique metabolism of cancer cells opens doors for possible cancer treatments including targeting lactate dehydrogenase and TCA intermediate production (58).

8. Treatment of Cancer

Cancer can be treated by surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy (in-cluding monoclonal antibody immunotherapy, and synthetic lethality. The choice of therapy depends upon the location and grade of the tumor, the stage of the disease, as well as the general state of the patient. Cancer genome sequencing helps in determining the best therapy for the cancer. Currently, it is estimates that two in five people will have cancer at some point in their lifetime [59]. Complete removal of the cancer surgically without damage to the rest of the body, would be the ideal goal of treatment and in practice. However, the tendency of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis, often limits surgery's effectiveness. Moreover, chemotherapy and radiotherapy can have a negative effect on normal cells. Thus, cure with no negligible adverse effects may be accepted as a practical goal in some cases. Besides curative intent, practical goals of therapy can also include: a) suppressing the cancer to a sub-clinical state and maintaining that state for years of good quality of life, and b) palliative care without curative intent for advanced metastatic can-cers. Because cancer refers to a class of diseases [60], it is unlikely that there will be a single cure for it, then there will be a single treatment for all infectious diseases [61]. Angiogenesis inhibitors were once thought to have potential as a "silver bullet" treatment that is applicable to many types of cancer, but this has not been the case in practice [62].

9. Cancer Prevention

Cancer prevention is the practice of taking active measures to decrease the incidence of cancer and mortality. Globalized cancer prevention is regarded as a critical objective due to its applicability to large populations, re-ducing long term effects of cancer by promoting proactive health practices and behaviors, and its perceived cost-effectiveness and viability for all socioeconomic classes [63]. The practice of prevention depends upon both individual efforts to improve lifestyle and seek preventive screening, and on socioeconomic or public poli-cy issues related to cancer prevention [64].

The majority of cancer cases are due to the accumulation of environmental pollution being inherited as epige-netic damage and many, but not all, of these environmental factors are controlla-

ble lifestyle choices [65]. Greater than a reported 75% of cancer deaths could be prevented by avoiding risk factors such as: tobacco, oveweight/obesity, an insufficient diet, physical inactivity, alcohol, sexually transmitted infections, and air pol-lution [50, 66]. Not all environmental causes are controllable, such as naturally occurring background radiation, and other cases of cancer are caused through hereditary genetic disorders. Current gene editing techniques un-der development may serve as preventive measures in the future [67]. Future preventive screening measures can be additionally improved by minimizing invasiveness, and increasing specificity by taking individual biologic make up into account, also known as "population-based personalized cancer screening [67]. It is recommended that experimental germline genome editing scenarios can proceed if: a) it is restricted to preventing transmission of a serious disease or condition, b) the edit is a modification to a common DNA sequence known not to be as-sociated with disease, and c) the research is conducted under a stringent set of ethical and regulatory require-ments. Crossing the red line of germline genome editing raises important bio-ethical issues, most serious concern about the potential negative impact on individuals with disabilities [68].

Unlike screening for large numbers of mRNA genes, a modest number of microRNAs is used to differentiate cancer from normal, and unlike mRNA, microRNAs in stool and blood remain largely intact and stable for de-tection [69]. Therefore, microRNAs are better molecules to use for developing a reliable noninvasive diagnostic screen for cancer, such as colon cancer, since we found out that: a) the presence of Escherichia coli does not hinder detection of microRNA by a sensitive technique such as qPCR, as the primers employed are selected to amplify human and not bacterial microRNA genes, and b) the microRNA expression patterns are the same in primary tumor, or in diseased tissue, as in stool and blood samples. The gold standard to which microRNA test is to compare to, should be colonoscopy, which is obtained from patients' medical records, as well as the cheaper immunohistological (IHC) FOBT screen, currently used in annual checkups, for comparison with microRNA re-sults [70]. Although exosomal RNA will be missed when using restricted extraction of total RNA from blood or stool [71], a parallel test could also be carried out on the small total RNA obtained from noninvasive stool or seminvasive blood samples, and the appropriate corrections for exsosomal loss can then be made after the tests are completed. A microRNA quantification workflow is presented in (Figure 1).

10. Clinical Significance of Micrornas in Human Cancer: Their Strengths & Weaknesses

When microRNA expression becomes dysfunctional, this could induce the dysregulation of gene expression, which in turn could result in disease. In microRNA profiling, one is often faced with two opposing scenarios. A promising scenario is that microRNA profiles show promise in biomarker discovery. A disappointing scenario is that microRNA profiles generated by different research teams do not always overlap, which does not inspire convincing confidence that the outcome could lead to clinically useful assays [72]. Part of the problem is that microRNA molecules consist of short nucleotide sequences (18 to 26 nt), which pose considerable challenges to probe design, and to label selection. Different microRNAs have widely varying melting temperatures for anneal-ing reactions; are present in concentrations that vary by orders of magnitude, which makes it hard to distinguish them from microRNA precursors and variants arising from post-transcriptional modifications, making accurate identification and quantification of microRNA molecules to be inherently challenging, as these molecules are relatively unstable when compared to the stable mRNA molecules [72].

An additional complication is that microRNAs are found in different contexts in tissues, in isolated cells, in extracellular vesicles, such as for example: exosomes; and they also are also bound with proteins in bio-fluids. Moreover, different microRNA samples call for different kinds of extraction techniques, and it is found that extraction techniques of the same kind vary significantly, which often leads to opposing results. Other sources of variability are observed in different microRNA measurements, and in the interpretation of the produced re-sults. On the other hand, microRNAs are relatively stable in various sample types, such as for example: liquid biopsy samples, as well as fresh and fixed tissue samples, which raises the possibility that stable microRNA mol-ecules can be accurately quantitated in order to produce disease signatures that can be uniquely recognized for different microRNA in different tissue samples. It is, however, very important to overcome variability be-tween samples and microRNAs tested, standardize microRNA profiling protocols, reconcile findings among various research groups, and to also develop reliable applications that deliver accurate diagnostic, prognostic and response to therapy findings, in order to fulfill the potential of microRNA to serve as biomarkers for various human diseases. Literature on the subject, have shown that microRNA profiles differ among cancers, depending on the method used to assay these molecules, as well as on individuals' health status, as have been shown in many published studies in the open literature [72].

10.1. MicroRNA Quantification & Profiling Basics

MicroRNA quantifications and profiling, may be studied from many sources, including a magisterial article that appear in published litterateurs [73]. This particular article outlined microRNA profiling workflows for dif-ferent sample types, emphasized the importance of quality control, and summarized the main microRNA quanti-tation techniques. The workflows encompassed: a) Purification techniques (such as gel electrophoresis, immuno-precipitation, and laser capture microdissection), b) Quality control checks (such as measuring spike-in oligo concentrations or housekeeping RNA expression, spectrophotometric analysis, or auto-

mated capillary electro-phoresis), and c) MicroRNA measurement techniques (such as reverse transcription quantitative PCR-based methods, hybridization-based methods, and RNA sequencing). In a study that employed RNA-seq for quantita-tive profiling of small RNAs (e.g., microRNAs, piRNAs and snoRNAs) in diverse sample types, including iso-lated cells, tissues and cell-free biofluids to systematically test the accuracy and reproducibility of the currently used small RNA-seq library preparation methods, results were reported from a consortium of nine labs that in-dependently sequenced reference samples of synthetic small RNAs, and human plasma-derived RNA. Three commercially available library preparation methods using adapters of defined sequence, and six methods that employed adapters with degenerate bases were assessed. Protocol- and sequence-specific biases were identified, including biases that reduced the ability of small RNA-seq to accurately measure adenosine-to-inosine editing in microRNAs. Results showed that these biases were mitigated by library preparation methods that incorporate adapters with degenerate bases. MicroRNA relative quantification between samples using small RNA-seq was shown to be accurate and reproducible across several laboratories and varying methods [73].

10.2. Tools and Reagents Critical for MicroRNA Research

To get a glimpse of how tool providers are important in securing reproducible results for microRNA quantifi-cation, outlined below are tools and reagents employed in microRNA research, and a discussion of their limita-tions, and ways to improve on them to produce consistent results across different laboratories. Effective, safe and reproducible microRNA purification tools are critical for obtaining reproducible microRNAs results. Natu-rally, it would be difficult to perform accurate and consistent microRNA biomarker discovery if there is a purifi-cation bias for particular miRNAs, or if there are inefficient dissociations of the microRNA from protein com-plexes from tissues, plasma, serum, and also from exosomes. Automation of these workflows allow for scaling of throughput to suit the needs of different uses. For example, in situ hybridization detection of mi-croRNAs, proper sample preparation and probes with good signal-to-noise ratios and specificity are critical [74].

Numerous methods have been adopted for the detection and characterization of small RNAs, which is quite challenging due to their short length and low level of expression. These methods include molecular biology methods such as northern blotting, real-time RT-qPCR, digital absolute PCR, hybridization to microarrays, clon-ing and sequencing, as well as single cell microRNA detection by microscopy with an in-situ hybridization (ISH) method. Several companies offer a variety of Locked Nucleic Acid (LNA) probes for in situ detection of mi-croRNAs, along with protocols and reagents for sample processing. For PCR and sequencing technologies, the first limiting factor is the isolation of sufficient quantities of high-quality total RNA from different samples. For some of these molecular technologies, the next critical factor is to have efficient amplification linkers or good stem-loop primers to accurately convert the miRNA into a cDNA that can be used for analysis [75].

RNAscope is a recent technique for elucidating the spatial resolution of microRNA transcripts, and provides important insight into potential gene function, probe design and propriety amplification technology, which pro-vides for simultaneous single molecule detection of individual microRNA and its target gene, allowing for rapid and sensitive detection of noncoding RNA transcripts in frozen tissue sections [76].

MicroRNA ISH is a very challenging technique because of the unique microRNA features, such as small size, sequence similarity among various microRNA family members, and low tissue-specific or development-specific expression levels. Therefore, standard ISH protocols were modified to improve microRNA detection in various types of cell lines and tissues, as well as whole embryos, including modifications at each step of the ISH protocols, probe design, cell fixation and permeabilization, hybridization, post-hybridization steps including washing, optional signal amplification and detection. Locked Nucleic Acid (LNA) modification, which has an additional bridge connecting 4′C and 2′O atoms, is considered the gold standard in RNA FISH [77].

For single molecule detection, different types of sequence and signal amplification techniques have been used to provide better resolution. Enzymatic signal amplification methods are used mainly to image low-abundant miRNAs in tissues. The most commonly used system for sequence amplification is Rolling Circle Ampli-Fication (RCA) or visualizing individual microRNAs [78, 79].

Global microRNA expression analysis in tissues is typically performed with the help of chromogenic enzyme-based detection methods; however, for more precise small RNA localization studies, fluorescent imaging is more suitable. Sequence and signal amplification methods significantly increase the signal-to-noise ratio and enable the detection of low abundant RNAs. However, neither the RCA-based techniques, nor fluorescent (ELF) sig-nal amplification methods are recommended when quantitative determination of small RNA levels are required. Therefore, choosing the right specific ISH variation is quite important. LNA nucleotides are incorporated into DNA probes, which leads to the formation of hybrid LNA/DNA probes. LNA/DNA probes have been shown to be highly beneficial in microRNA detection because of a short hybridization time, high efficiency, discriminato-ry power and a high melting temperature of the microRNA: probe complex. The minimal length of the LNA/DNA probe has been determined to be 12 nucleotides [80], and these probes usually contain 30% LNA nucleotides. Despite their advantages, it is instructive to know that these probes are expensive, and they can generate strong background signals, resulting in a low signal-to-noise ratio for low abundant microRNA [81].

Despite the success of the ISH adaptation for small RNA detection and subcellular localization, the various in situ hybridization techniques also have their limitations. Major limitations are: a) they capture small RNAs in the cell only at one point of time, and b) they are incapable of distinguishing between RNA functional and non-functional states. The latter may include RNA that is awaiting its cellular function, or RNA stored for degrada-tion. The only difference between the functionally active and inactive small RNAs might be the type of proteins or transcripts with which they interact while active or stored. The co-localization of microRNA with target mRNAs, or Ago proteins, could partially solve the problem. Another solution is using an analysis that captures the downstream effect of target transcript cleavage, or degradation. There is however, no system available for live RNA imaging capable of labeling small RNAs [82]. The MS2 system was adapted for imaging microRNA primary precursors, i.e., pri-microRNA [83, 84], but not their processing products, i.e., mature microRNAs. The first attempt to monitor microRNAs in living cells, has employed a carbon nitride nanosheet probe [85]. An ad-ditional challenge is the ability of the ISH method for single molecule detection. Methods developed for single mRNA transcript detection [86-88] cannot be used for small RNA imaging because these methods are typically based on the use of multiple probes that target different regions of a single mRNA. Signal amplification tech-niques, which were adapted for small RNA analysis, enable the visualization of low abundant microRNAs; however, it has not yet been established whether these methods are capable of precise single molecule detection [89].

Nanotechnology was recently introduced in the field of microRNA ISH to improve on the detection of small RNAs. Metal nanoshells composed of silica spheres with encapsulated Ru(by)32+ complexes as cores, and thin silver layers as shells, have been used for the detection of low-abundant microRNAs. The metal nanoshells are based on near-field interactions between organic fluorophores and metal nanoparticles. The use of nanoshells resulted in overcoming many of the difficulties that have been specific to organic fluorophores, including re-duced photoblinking, increased photostability and intensity, as well as a noticeable increased lifetime of the or-ganic fluorophores in, comparison to the lifetime of cellular autofluorescence [90].

However, it should be stressed that the system needs to be optimized in order to provide for a much better penetration of the nanoshells through the cell membrane, mobility in the cells, as well as the high specificity towards the microRNA target(s) [90].

Commercial assays for the detection of several microRNAs using in situ hybridization and fluorescence in situ hybridization are also available [91, 92]. A branched DNA system for signal amplification has been proposed to enable microRNA quantitative analysis]. The system was also found efficient in the detection of exogenous siRNA molecules [93].

An important step in an ISH experiment is probes design. Different probe types have different properties and detection options that enable selection of suitable solutions for many applications. Two types of probes are available: a) linear probes that are directly labeled with fluorophore or ligand, and b) probes that permit for sequence amplification [94].

10.2.1. Direct Labeled Probes

In standard ISH, probes composed of DNA or RNA nucleotides are commonly used. Because unmodified DNA and RNA probes have relatively poor binding affinity to target sequences [94], therefore several modifica-tions have been made to improve on their properties. First, was Locked Nucleic Acid (LNA) modification, which is the gold standard in RNA FISH. LNA nucleotides, referred to as "locked" RNA, have an additional bridge connecting 4′C and 2′O atoms. LNA nucleotides are incorporated into DNA probes, which leads to the formation of hybrid LNA/DNA probes. LNA/DNA probes are beneficial in microRNA detection because of a short hybridization time, high efficiency, discriminatory power and a high melting temperature of the mi-croRNA: probe complex. The minimal length of the LNA/DNA probe is twelve nucleotides [95], and these probes usually contain 30% LNA nucleotides. These probes are expensive and can generate strong background signals, resulting in a low signal-to-noise ratio for low abundant microRNA [96].

Therefore, other modifications were also proposed, including 2′fluoro-modified RNA (2′F RNA), morpho-lino, zip nucleic acids (ZNA) [97], N, N-diethyl-4-(4-nitronaphthalen-1-ylazo)-phenylamine (ZEN) [98], and 2′O-Methyl (2′OMe) RNA modification. compared to DNA probes, 2′OMe RNA probes have faster hybridiza-tion kinetics, and the ability to bind targets under standard conditions [99]. The combination of 2′OMe RNA and LNA modifications (in a 2:1 ratio) resulted in improved specificity and stability of the probe: RNA duplex in comparison to the LNA/DNA probe [100]. Specificity of the system could be improved by shortening the probe length to 19 nucleotides. Better results were obtained without use of RNA blockers in the hybridization step [100]. 2′F RNA nucleotides incorporated in the DNA probes ensure increased binding to the target and bet-ter nuclease resistance [101]. Morpholino modifications used to inhibit translation, modify splicing patterns of the primary transcript, or block microRNAs could also be used to detect microRNAs because of their high sta-bility [102].

10.2.2. Probes Used with Sequence Amplification Methods

Padlock probes are used for sequence amplification and detection of microRNAs, mRNAs and DNA se-quences, with the high sensitivity and specificity of single nucleotide discrimination [103], making them suited for allele-specific FISH [104]. Linear DNA probe, after annealing to the specific sequence with 5′ and 3′ arms, is circularized by DNA ligase. The circularization enables further signal amplification by rolling circle amplification (RCA). RCA

uses microRNA molecule as a primer, and elongates the sequence using circular probe as a tem-plate. Detection of the RCA product is accomplished by the use of probes complementary to the sequence am-plified on the template of the padlock probe central sequence. Other probes used in microRNA ISH in combi-nation with RCA, are circular DNA probes, which are obtained in vitro with the use of padlock probes, ligation probes, and DNA ligase, and are then hybridized to the target sequence in cells as circular molecules [105].

Another probe used together with RCA is a "seal probe", with an adjustable toe hold inside its loop. These probes have the ability to change their structure. The initial dumbbell shape of the probe is changed into a circu-lar form, when the target microRNAs bind into the toehold domain of the probe, leading to reaction inhibition, and an RCA reaction is initiated. This method is called "toehold-initiated rolling circle amplification (TIRCA)", and is a combination of both toehold-mediated strand displacement (TMSD) and an RCA. The length of the toehold defines the stability of the probe, and is considered to be the most important factor for detecting mi-croRNAs with TIRCA. Increasing length of the toehold, leads to decreased stability of the seal probe and selec-tivity of TIRCA. Advantages of using TIRCA is reduced loss of microRNA molecules, because the detection process is conducted at a physiological temperature, short imaging time, and with high sensitivity and specifici-ty, which is higher than that of a padlock probe-based RCA reaction [106]. Sequence amplification is also achieved by RT in situ PCR in what is known as the ultramer extension method, which is based on the use of longer probes while microRNA is acting as a primer, as in the RCA method. The probe contains sequences com-plementary to microRNA and a series of 20 nucleotide sequences at the 5′ end. During in situ PCR, digoxigenin (DIG)-labeled nucleotides are incorporated to the PCR product, and are detected with antibodies [107].

MicroRNA ISH based on enzymatic detection was also implemented in molecular diagnostics using tissue microarrays [108, 109], which enables high throughput fast detection of specific microRNAs in many tissue samples simultaneously, using both LNA/DNA probes and TSA labeling [110]. Automation in multiplex mi-croRNA detection was also implemented using the same detection system [111]. Detection of microRNA ISH can be enzyme-based, in which nonradioactive haptens, combined with probes, are detected by histochemical enzymatic reactions after application of enzyme-conjugated anti-hapten antibodies. Alkaline Phosphatase (AP) is most commonly used, with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) as a sub-strate [112].

The Tyramide Signal Amplification System (TSA), or the Catalyzed Reporter Deposition Method (CARD), is a similar approach. Currently LNA probes labeled with digoxigenin are mostly used in the hybridization step. In the TSA/CARD system, the Horseradish Peroxidase (HRP)-tagged anti-digoxigenin antibodies recognize di-goxigenin moieties on probes. Next, HRP substrates, i.e., cyanine 5 (Cy5), cyanine 3 (Cy3) or fluorescein-conjugated tyramides, are converted to highly reactive, short-lived radicals by HRP, which bind covalently to tyrosine residues located nearby [113]. The TSA/CARD system improved the sensitivity up to 1000-fold com-pared to its early version [114]. Another detection method is Enzyme-Labeled Fluorescence Signal Amplification (ELF), in which short, pro-luminescent substrate cleavage is carried out by phosphatase, followed by precipita-tion of a bright, yellow-green fluorescent product that is highly photostable, and gives a much brighter signal compared to probes directly labeled with fluorophores [115], with a dynamic range spanning over three orders of magnitude [116].

Nanotechnology was introduced in microRNA ISH to improve on the detection of small RNAs. Metal nanoshells composed of silica spheres with encapsulated Ru(bpy)32+ complexes as cores, and thin silver layers as shells, have been employed for the detection of low-abundant microRNAs. Metal nanoshells are based on near-field interactions between organic fluorophores and metal nanoparticles. Nanoshells reduced photoblinking, in-creased photostability and intensity, as well as showing an increase in the lifetime of the organic fluorophores (117). Nevertheless, the system needs optimization in order to provide better penetration of the nanoshells through the cell membrane, induce mobility in the cells, and provides high specificity toward the microRNA tar-get [89]. Commercial assays for the detection of several microRNAs using in situ hybridization and fluorescence in situ hybridization are also available [118, 119], such as a branched DNA system for signal amplification to en-able microRNA quantitative analysis [120], which was shown to be also efficient in the detection of exogenous siRNA molecules [89].

10.2.3. Probe Specificity

While in RNA FISH, probes targeting different parts of the mRNA can be used to confirm probe specificity; however, this approach is not applicable to microRNAs due to their short length. Probes complementary to microRNA analogs from different organism or even different kingdoms can be used as negative control; for mammalian microRNA detection, probes against plant specific microRNA may be used. This approach may be applied only if microRNA analogs sufficiently differ in sequence [121].

Another negative control is the use of probes against microRNA that is not expressed in the analyzed tissue [122], or the use of scrambled probes. The scrambled probe should be checked not only for specificity against microRNA population but also against the whole transcriptome. In some experiments, probes similar in sequence to microRNA-specific probes but containing two or three mismatches with the target sequence was used [123]. As a positive and negative control, microRNA-specific probes can be used to label different tissues that were shown to exhibit this microRNA

expression or not, respectively [122].

To exclude false positive results caused by cells or tissue autofluorescence, control experiments without any probes are used [124]. Moreover, non-hybridization-based interactions are excluded by the treatment with unla-beled probes prior to hybridization with labeled probes [125]. Hybridization with probes that were already suc-cessfully tested can serve as positive controls for adequate experimental conditions and good RNA quality, e.g., snRNA U6 as a target is commonly used [126]. Interactions with DNA are excluded by DNase treatment, while interactions with RNA are confirmed by RNase treatment prior to hybridization [89].

10.2.4. Limitations of The Tools and Reagents Currently Used in Microrna Research

In this section, we look at specific examples of commonly employed technologies and discuss the extent of their limitations. The current standard method for purification is exosomes ultracentrifugation method. Alt-hough this method is reliable, it is also tedious and time consuming, and it is not amenable to high throughput. Reagents that can be used to precipitate exosomes without ultracentrifugation would provide faster and au-tomatable exosome purification. However, there appear to be differences in microRNA expression based on the exosome purification method. Additionally, most microRNA purification workflows currently on the market are completely manual. It is difficult to scale this workflow to large biomarker discovery or research projects.

10.2.4.1. Exosome Isolation Methods

Extracellular vesicles, found in all biofluids, include exosomes (30 nm to 150 nm) from endosomes/ mul-tivesicular bodies, and macrovesicles (150 nm to 1000 nm) from the plasma membrane. Multiple biotech compa-nies are exploiting exosomes as a delivery modality [127]. Various methods for the isolation of exosomes from biological fluids have been developed. They include centrifugation, chromatography, filtration, polymer-based precipitation and immunological separation. Recent technical improvements in these methods have made the iso-lation process faster and easier. Contamination of isolated exosomes with non-exosomal particles, such as apop-totic bodies, small apoptotic vesicles, exomeres, and lipoproteins can cause wrong conclusions about biological activities of obtained exosomes and therefore should be avoided [128]. Exosomes from different specimens can possess different protein/ lipid and luminal contents and different sedimentation characteristics. For example, exosomes from adipose tissue contain high lipid content, and necessitate adjustment in their isolation methods [129]. If exosomes are to be isolated from cultured media, one very important consideration is to use either se-rum-free media or exosome-free fetal bovine serum. See (Table 1).

As more microRNA markers are associated with different diseases and become important in diagnostics, the need to detect more microRNA markers in small samples is increasing. For several PCR-based technologies, it is not easy to distinguish microRNAs that may differ by a single nucleotide. In addition, the quantity of high-quality RNA that can be isolated from various body fluids (urine, sera, other body fluids) can be too low to identify microRNAs at low concentrations accurately [130-132]. When vital microRNA markers can vary by a single nucleotide, these factors can be a serious limitation for some diagnostic applications [133].

11. Use of Various Technologies Such as Northern Blotting, Single Cell Microscopy Detection, Microarray, Polymerase Chain Reaction (RT-Qpcr & Absolute Digital PCR), Gene Sequencing (NGS Tests for The Quantitative Detection of Cancer-Causing Micrornas in Diversified Body Samples

We have shown that we can routinely and systematically be able to extract a high quality small total RNA containing microRNAs from a small number of Laser Capture Micro Dissected (LCM) cells from tissue [134], co-lonocytes isolated from human stool, or circulating blood using commercially-available kits (RNeasy isolation Kit®) from Qiagen, Valencia, CA, USA, followed by another kit from Qiagen “The “Sensiscript RT Kit”. We can then quantify these microRNAs using various quantification technologies [135-140].

11.1. Next-Generation Sequencing (Nsg) Technologies

The 1977 chain-termination method of Sanger, commonly known as Sanger's dideoxy sequencing [141]. has been partly supplanted by other more cost effective next-generation sequencing technologies that provide higher throughput, but at the expense of read lengths. The Sanger method is based on DNA polymerase-dependent synthesis of a complementary DNA strand in the presence of 2'-deoxynucleotides (dNTPs) and 2',3'-dideoxynucleotides (ddNTPs) that serve as nonreversible synthesis terminators when ddNTPs are added to the growing oligonucleotide chains, resulting in truncated products of varying lengths, which can subsequently be separated by size on polyacrylamide gel electrophoresis. Advances in fluorescence detection have allowed for combining the four terminators into one reaction, using fluorescent dyes of different colors, one for each of the four ddNTP. Furthermore, the original slab gel electrophoresis was replaced by capillary gel electrophoresis, ena-bling better separation. Additionally, capillary electrophoresis was replaced by capillary arrays, allowing many in vivo amplified fragments samples cloned into bacterial hosts to be analyzed in parallel. Moreover, the develop-meant of linear polyacrylamide and polydimethylacrilamide allowed for the reuse of capillaries in multiple elec-trophoretic runs, thereby increasing the sequencing efficiency. These and other advances of the sequencing tech-nology have contributed to the relatively low error rate, long read length and robustness of modern Sanger se-quencers. For example, the high throughput automated Sanger sequence instrument from Applied Biosystems (ABI 37730xl) has a 96 capillary array

format that produces ≥900 PHRED 20 bp (a measure of the quality of identification of the nucleobases generated by sequencing) per read, for up to 96 kb, for a 3 h run [142].

The 454 Roche instrument was the first next generation sequencer released to the market that circumvents the lengthy, labor intensive and error-prone technology by using in vitro DNA amplification known as emulsion PCR, where individual DNA fragment-carrying streptavidin beads, obtained by the shearing the DNA and at-taching the fragments to beads using adapters, which are captured into separate emulsion droplets that act as individual amplification reactors, producing ~ 107 clonal copies of a unique DNA template per bead. Each tem-plate-containing bead is then transferred into a well of a picotiter plate, which allows hundreds of thousands of clonally related templates of pyrosequencing reactions to be carried out in parallel, increasing sequencing output [143].

The sequence of DNA template is determined by a program, which corresponds to the correct order of chemiluminescently incorporated nucleotide as the signal intensity is proportional to the amount of pyrophos-phate released. The pyrosequencing approach is prone to errors resulting from incorrectly estimating the length of homopolymeric sequence stretches (or indels). The Roche 454 platform, a widely used next generation sequencing technology, is capable of generating 80 Mb - 120 Mb of sequence in 200 bp - 300 bp reads in a 4 h run The Illumina/Solexa approach achieves cloning-free DNA amplification by attaching a ssDNA fragment to a solid surface, known as a single molecule array, or free cell, and performing solid phase bridge amplification of single molecule DNA templates in which one end of single DNA molecule is attached to a solid surface by an adapter; the molecule is subsequently bend over and hybridized to complementary adapters, creating a bridge, which serves as a template for the synthesis of complementary strands. Following the amplification, a flow cell containing more than 40 million clusters, each cluster composed of ~ 1000 clonal copies of a single template molecule is produced. Templates are sequenced in massively parallel manner using a DNA sequencing-by-synthesis approach that employs reversible terminators with removable fluorescent moieties and DNA polymer-ases capable of incorporating these terminators into growing oligonucleotide chains. The terminators are labeled with fluors of four different colors to distinguish among the different bases at the given sequence position, and the template sequence of each cluster is deduced by reading off the color at each successive nucleotide addition step. Although Illumina technology seems more effective at sequencing homopolymeric stretches than pyrose-quencing, it produces shorter sequence reeds, and thus cannot resolve short sequence repeats. Moreover, substi-tution errors have been noted in this platform due to the use of modified DNA polymerases and reversible ter-minators. The 1G Illumina genome analyzers generates 35 bp reads per run in 2 days to 3 days [143].

Massively parallel sequencing (MPS) by hybridization-ligation supported in the oligonucleotide ligation and detection system SOLiD from Applied Biosystem is based on the polony sequencing technique [144]. Libraries begins with an emulsion PCR single-molecule amplification step, followed by transfer of the products onto a glass surface, where sequencing occurs by sequential rounds of hybridization and ligation with 16 dinucleotide combinations labeled by four different fluor dyes. Each position is probed twice and the identity of the nucleo-tide is determined by analyzing the color resulting from two successive ligation reactions. The two base encod-ing schemes allow the distinction between a sequencing error and a polymorphism (an error would be detected in only one reaction, whereas a polymorphism would be detected in both). The 1-3 GB SoLiD generates 35 bp reads per an 8day run (145). Table 2 depicts available DNA sequencing technologies.

11.2. Microarray Employed Technologies

For microarray studies, we employed Affymetrix Gene Chip Micro 3.0 Array (Affymetrix, Inc, Santa Clara, CA, USA), which provides for 100% miRBase v17 coverage [http://ww.mirbase.org] by a on color approach. The microarray contains 16,772 entries representing hairpin precursor, expressing 19,724 mature microRNA products in 153 species, and provides >3 log dynamic range, with 95% reproducibility and 85% transcript detec-tion at 1.0 amol for a total RNA input of 100 ng. [146]. Global microarray expression studies [147, 148], have shown similarity in expression between stool, plasma and tissue [147]. Microarray studies in stool samples ob-tained from fifteen individuals (three controls, and three each with TNM stage 0-1, stage 2, stage 3, and stage 4 colon cancer) showed 202 preferentially expressed microRNA genes that were either increased (141 mi-croRNAs), or decreased (61 microRNAs) in expression [135].

11.3. Relative and Absolute Polymerase Chain Amplification Reaction (Qpcr & Dpcr) Quantification Technologies

To be able to screen several microRNA genes using the proposed PCR technology in a sequence-specific manner, in which a cDNA preparation [149] can assay for a specific microRNA, we have employed in our work a sequence-specific stem-loop RT primers designed to anneal to the 3'-end of a mature microRNA, which result in better specificity and sensitivity compared to conventional linear ones [150]. This step was followed by a SYBR Green®-based real-time qPCR analysis in which a forward primer specific to the 5'-end of the mi-croRNA, a universal reverse primer specific for the stem-loop RT primer sequence, and a 5'-nuclease hydrolysis probe -TaqMan minor grove binding (MGB) probe --matching part of the microRNA sequence and part of the RT primer sequence-- was employed in our Lab, using a standard TaqMan PCR kit from Applied Biosystems on a Roche'sLight Cycler (LC) 480 instrument, which employed the "E-method" to calculate the relative expres-sion of microRNA genes in modified RT-qPCR studies [151].

It should be emphasized that the Roche's LC-480 PCR instrument [152] employs a nonuser influenced method for high throughput measurements, using second derivative calculations and double corrections [153]. One correction utilizes the expression levels of a housekeeping gene of an experiment as an internal standard, which results in reduced error due to sample preparation and handling, and the second correction uses reference expression level of the same housekeeping gene for the analyzed expression in colonocytes or plasma, which avoids the variation of the results due to the variability of the housekeeping gene in each sample, especially in experiments that employ different treatments [154].

11.3.1. Method for Qpcr Quantification, Normalization, And Quality Control (QC) Issues

The comparative cross point (CP) value (or E-method) [151] was employed, utilizing the Light Cycler (LC) Quantification Software™, Version v4.0 or 3.5 [152] for Roche LC PCR instruments (Mannheim, Germany) for the semi-quantitative PCR analysis. The method employs standard curves in which the relative target concentra-tions is a function of the difference between crossing points (or cycle numbers) as calculated by the second de-rivative maximum [153], in which the Cycler's software algorithm identifies the first turning point of the graph showing fluorescence vs. cycle number to calculate the expression of microRNA genes automatically without user's input, with a high sensitivity and specificity. A CP value corresponds to the cycle number at which each well has the same kinetic properties. The CP method corresponds to the 2-ΔΔCT method used by other PCR in-struments, although the latter method produces reliable quantitative results only if the efficiency [E=10-1/slope] of the PCR assay for both target and reference genes are identical and equal to 2 (i.e., doubling of molecules in each amplification cycle); for example, if well A1 has a CP value of 15 and well A2 has a CP value of 16, we deduce that there was twice as much of the gene of interest in well A1. A 10-fold difference is shown by a dif-ference of ~ 3.3 CP value. It is impossible to compare experimental values obtained between different primer pairs. The CP method compensates for difference in target and reference gene amplification efficiency, either within an experiment, or between many different types of experiments [153].

It is also essential to normalize the data to a "reference" housekeeping internal standard gene (e.g., endoge-nous reference genes RNU6 genes RNU6A and RNU6B, SNORD genes SNORD43, SNORD44, SNORD48, SNORA74A) or microRNA normalizers (e.g., microRNA 16, microRNA-191), or in some cases against several standards (154-157) because the total input amount may vary from sample to sample when doing relative quan-tification [158]. To ensure that microRNA quantification is not affected by the technical variability that may be introduced at different analysis steps, synthetic nonhuman spike-in microRNA have been used to monitor RNA purification and RT efficiencies. The C. elegans cel-microR-39, cel-microR-54, the synthetic microRNAs Quan-to ECI and Quanto EC2, and the simian virus gene SV40 have been used; these exogenous microRNAs are usu-ally added to samples before the RT step to avoid differences in template quality, or affect the efficiency of the RT reaction, and can eliminate deviation of the results, making results reliable, but does not correct for sampling deviation or quality of tissues, body fluid or extracellular vesicle samples. It has been proposed that the best normalization strategy is the one that employs a combination of exogenous and endogenous control mi-croRNAs because this combination compensates for differences in microRNA recovery and cDNA synthesis among samples [159-161].

Some studies used absolute data normalization and calculated microRNA expression using standard curves developed by synthetic microRNA and melting curves normalized per nanogram of the total input RNA for microRNA-221 and microRNA- 18a in 40 pairs of CRC tissue and 595 stool samples, a technical detection limits of 2 copies for microRNA-221 resulted in a Cq value of 42, and a technical detection limit of 5 copies for microRNA-18a resulted in a Cq value of 47, which were all assigned a value of 0, similar samples with no amplification of microRNA-221 or microRNA-18a as analyzed vy Volcano plots [162]. It should note, however, that values of CQ >40 are unreliable. Absolute normalization method is thus considered to be a reliable method only for samples with good RNA standardized quality [163]. To report "fold change" results, the LC software incorporates all those factors.

The CP method can normalize for run-to-run differences, as those caused by variations in reagent chemistry. For such normalization, one of the relative standards must be designated a "calibrator" for the target and for the reference genes, which can be any of our healthy control stool sample. These calibrator(s) can then be used re-peatedly in subsequent runs to guarantee a common reference point, allowing for comparison of all experiments within the series. If necessary, the 2-ΔΔCT can be calculated by instrument's software if samples are properly labeled; the 2-ΔΔCT calculations can also be set up manually. To determine fold change for a particular un-known cancer stool or blood sample that has a target gene CP value of 10, one needs three additional values: a) The reference gene CP value of that same unknown stool sample/ cancer stool sample, b) the target gene CP for the calibrator sample/ normal stool, and c) the reference gene CP for the calibrator sample/ normal stool or blood [163]. In all PCR reactions, strict attention must be given to quality control (QC) procedures, and as the field has matured, guidelines on reporting qPCR data known as Minimum Information For Publication Of Quanti-Tative Real-Time PCR Expression (MIQUE), which is a set of guidelines that described the minimum information necessary for evaluating qPCR experiments that include a checklist to accompany the initial submission of a manuscript to the publisher providing all relevant experimental conditions and assay characteristics, so that re-viewers can assess the validity of the protocols used, as well as full disclosure of all

reagents, sequences and analytical methods employed to enable other investigators to reproduce results, with MIQE details published either in abbreviated form, or as an online supplement, in order to ensure the uniformity, reproducibility and reli-ability of the PCR reaction and data integrity [164].

11.4. Absolute Quantitative Digital PCR Approach

Digital PCR is a newer approach to microRNAs quantification that offers an alternate method to qPCR for absolute quantification, by partitioning a sample of DNA or cDNA into many individual, parallel PCR reactions; some of these reactions contain the target molecule (positive), while others do not (negative). A single molecule can be amplified a million-fold or more [165]. During amplification, TaqMan chemistry with dye-labeled probes is used to detect sequence-specific targets. When no target sequence is present, no signal accumulates. Follow-ing PCR analysis, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, without the need for standards or endogenous controls. In conventional qPCR, the sig-nal from wild-type sequences dominates and obscures the signal from rare sequences. By minimizing the effect of competition between targets, dPCR overcomes the difficulties inherent to amplifying rare sequences and al-lows for sensitive and precise absolute quantification of the selected microRNAs. Applied Biosystem QuantStudio™ 3D instrument, which we used in our research study, only performs the imaging and primary analysis of the digital chips. The chips themselves must be cycled offline on a Dual Flat Block GeneAmp® 9700 PCR System or the ProFlex™ 2x Flat PCR System. The QuantStudio™ 3D Digital PCR System can read the digital chip in less than 1 minute, following thermal cycling [165, 166].

The method generally allows for the analysis of one sample per chip; although duplexing allows for analysis of two targets per chip. Sample prep for digital PCR is no different than for real-time relative qPCR, when using the QuantStudio™ 3D Digital PCR System [165].

To figure out the concentration of cDNA stock from results, if one includes all of the necessary dilution fac-tors into the AnalysisSuite™ software, the software will give the copies/µL in the stock. There are 2 dilutions that one needs to consider: (a) The first is the dilution of the sample in the reaction, and (b) The second is the dilution of the stock that one makes before adding it to the digital PCR reaction. For example, if one wants to add 1 µL of a sample that has been diluted 1:10 from the stock [for example, if one adds 1 µL of his/her sample to a 16 µL (final volume) reaction, the dilution factor of the sample is 1:16 or 1/16=0.0625. Since the stock has also been diluted 1:10 (0.1), then one also needs to factor this in. The final dilution factor to enter into the soft-ware is then $0.0625 \times 0.1 = 0.00625$ (1:160). One can use either annotation to indicate the dilution factor in the AnalysisSuite™ software [166].

The use of a nanofluidic chip provides a convenient forward mechanism parallel to run thousands of PCR reactions. Each reaction well is loaded with a mixture of sample, master mix, and reagents for an Applied Biosystems TaqMan Assay reagents, Databases are individually analyzed to detect the presence (positive) or ab-sence (negative) of an endpoint signal. In order to account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model, which features a filter set that is optimized for the FAM™, VIC®, and ROX™ dyes, available from Life Technologies. The chips themselves are cycled offline on a Dual Flat Block GeneAmp® 9700 PCR System or on the ProFlex™ 2x Flat PCR System. The QuantStudio™ 3D Digital PCR System of Applied Biosciences can read the digital chip in less than 1 minute, following thermal cycling. The instrument allows for one sample per chip; although use of du-plexing will allow for analysis of two targets per chip. Sample preparation for digital PCR is also similar to that for real-time relative qPCR method, when using the QuantStudio™ 3D Digital PCR System [166].

To figure out the concentration of cDNA stock from results, the system operator must include the necessary dilution factors into the AnalysisSuite™ software, and the software is programed to automatically calculate the copies/µL in the stock. A workflow of the dPCR procedure by the QuantStudioTM 3D Digital PCR System is presented in (Figure 2). To obtain accurate results with digital PCR, the operator has several steps to follow: 1) A rough estimate of the concentration of the microRNAs of interest has to be first figured out, in order to make the appropriate dilutions, so that not too many partitions will get multiple copies, which would prevent an accu-rate calculation of the copy number of microRNAs of interest; 2) Non-template controls, and a RT negative con-trol must be set up for each microRNA of interest, when using a “primer pool method” for retro-transcription; 3) A chip-based dPCR method requires less pipetting steps, which reduces potential PCR contamination compared to another type of dPCR marketed by Bio-Rad Laboratories, thus called “Bio-Rad's droplet digital PCR”, which requires multiple pipette transfers that potentially increase the risk of contamination, and 4) the Quant StudioTM 3D chip has 20,000 fixed reaction wells, whereas Bio-Rad’s droplet digital PCR relies upon the generation of droplets; a step that could be extremely variable [167]. The digital MIQE guidelines on minimum In-formation for publication of quantitative digital PCR experiments have been published (168).

11.5. Innovation and Clinical Significance of the Dpcr-Microrna Stool Screening Approach

Innovation of our long-term research to quantitatively and accurately measure microRNA molecules in human stool samples to access the occurrence and for the extent progression of human colon cancer in affected individuals, lies in the collective use of

many technical methods, such as: immunoparamagnetic beads to cap-ture colonocytes from the harsh, noninvasive stool environment, whose extracted fragile total small RNA is sta-bilized shortly after stool excretion by commercial kits so it does not ever fragment, followed by a standardized analytical quantitative microRNA dPCR-chip profiling in non-invasive stool samples, which are neither labor intensive, nor require extensive sample preparation, to develop a panel of few stable microRNAs for the absolute quantitative diagnostic screening of early sporadic colon cancer (stage 0-1), that is cheaper, quantitative with higher sensitivity and specificity than any other colon cancer screening test on the market today. Isolation of colonocytes from stool samples is needed to provide a quantitative estimate of how our quantitative, sensitive and accurate microRNA method performs. Although we could miss exosomal RNA, a parallel test needs to be employed on microRNAs obtained from stool samples to compare the extent of loss when colonocytes are only used, and an appropriate correction for exsosomal loss can be made accordingly [169].

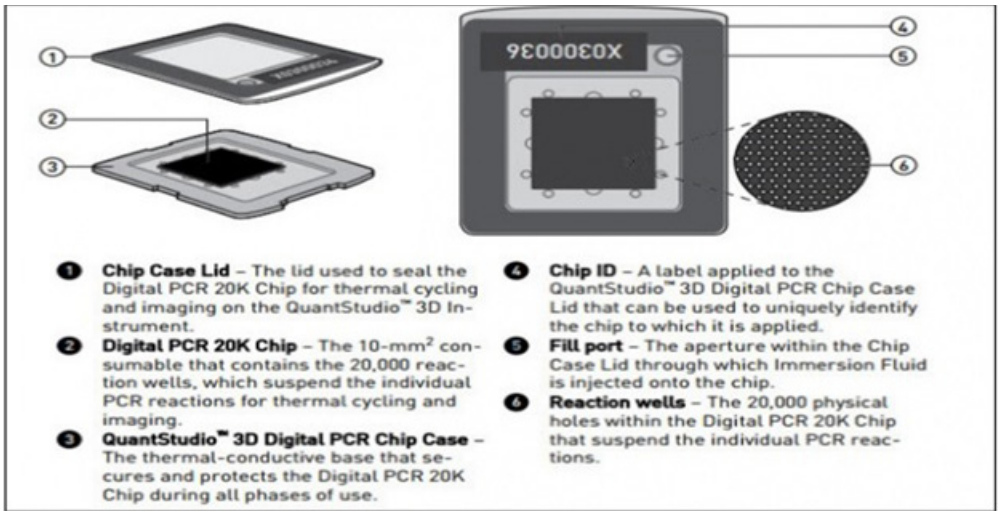

Figure 2: Diagram illustrating Quant StudioTM 3D Digital PCR System Chip; ChipCase Lid (1); Digital PCR 20 K 10 mm 2 nanofluidic v2 chip case and lid (2), which contains 20,000 reaction wells; QuantStudioTM 3D Dig-ital PCR Chip Case (3); Chip ID (4); Fill port (5); and Reaction wells, the 20,000 physical holes (6) that suspend individual PCR reactions. Modified from reference 167.

12. Micrornas' Measurements from Exosomes and Macrovesicles Extracted from Human Stool

MicroRNAs are resistant to ribonucleases present in stool, probably by inclusion in lipid or lipoprotein complexes in either macrovesicle (up to 1 μm), or in small membrane vesicles of endocytic origin known as exo-somes [(50-100 nm) [170-172]. The mechanism of release of microRNA from exosomes and macrovesicles' is un-clear, although an apoptotic delivery candidate is shed from cells during apoptosis [173]. Exosomes released from human, and murine mast cell lines were shown to contain mRNAs and microRNAs [174]. MicroRNAs in microvessicles were shown to function in cellular communication [175], regulate cellular differentiation of blood cells and certain metabolic pathways, and to modulate immune functions [176]. MicroRNA signatures of tumor-derived exosomes were shown to function as diagnostic markers in ovarian cancer, and tumor-derived mi-croRNA profiles and profiles of exosomal microRNAs were not significantly different [177]. If necessary, exo-somal microRNAs extracted from stool colonocytes by differential centrifugation, followed by filtration through 0.22 μm filters, total RNA extracted by Trizol & concentration measured at λ 280 [178].

13. Correlation Between Micrornas and Cancer

13.1. History and Nomenclature

The role of the small highly conserved, non-coding microRNA molecules that contain ~ 22 nucleotides long RNA sequences, found in plants, animals and viruses that function in RNA silencing and in post-translational regulation of gene expression, in cancer [179], following microRNA initial discovery in early 1993 by Victor Ambros and collaborators by classic forward genetics [180]., while working on the lin-4 gene, which controls the timing of the nematode worm Caenorhabditis. Seven years after the discovery of microRNA in 2001, the lin 4 and let-7 were found to be part of a large class of small RNAs' posttranscriptional regulators found in C. ele-gans, Drosophila and human cells and conserved across animal phylogeny [181], referred to herein now as mi-croRNAs, as shown in (Figure 3).

MicroRNA genes are generally present in the intronic regions, and function as the transcriptional control of the regulatory elements of other protein coding genes. Approximately 2200 microRNA genes exist in the mammali-an genome, from which more than 1000 belong to the human genome [182]. One third of the human genome is estimated to be regulated by microRNAs. The first

human disease associated with this deregulation of mi-croRNAs was chronic lymphocytic leukemia, and microRNA-181b was the biomarker for the progression of this disease [183]. Major differences exist between plant and animal microRNAs in the extent of gene regulation and sequence complementarity requirements for mRNA-microRNA base pairing [184]. All RNAs that do not yield coding proteins are called non-coding RNAs (ncRNA) (200 nucleotides or longer), and they contain mi-croRNAs, small nucleolar RNAs (snoRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs), and PIWI-interacting RNAs (piRNAs) (Figure 4) (185). MicroRNAs are involved in protein translation (Figure 5). The structure of the microRNA molecule is shown in (Figures 3 and 6).

MicroRNAs are abundant in mammalian cells and seem to target ~ 60% of human genes and also in other mammals (185). Many microRNAs are conserved evolutionary, implying that they have important biological functions [186]. In September 2012, Johnson & Johnson presented the 2012 Paul Janssen award for biomedical research to Drs. Victor Ambros and Gary Ruvkun for their collaborative discovery of microRNAs as central im-portant regulatory molecules, who play a pivotal role in gene expression, as well as in development.

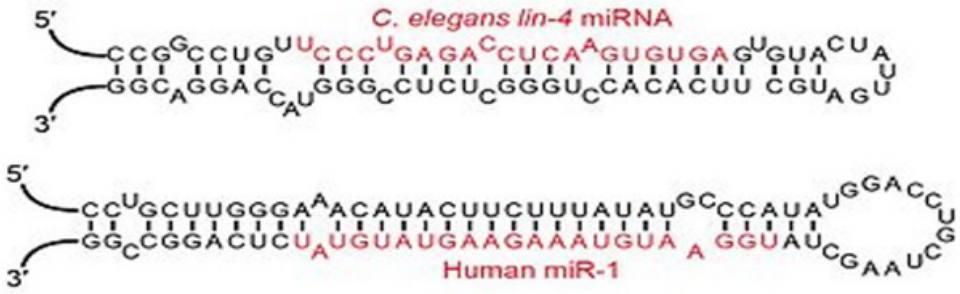

Figure 3: Mature nematode Caenorhabditis elegans lin-4 microRNA & human microRNA-1 stem loops

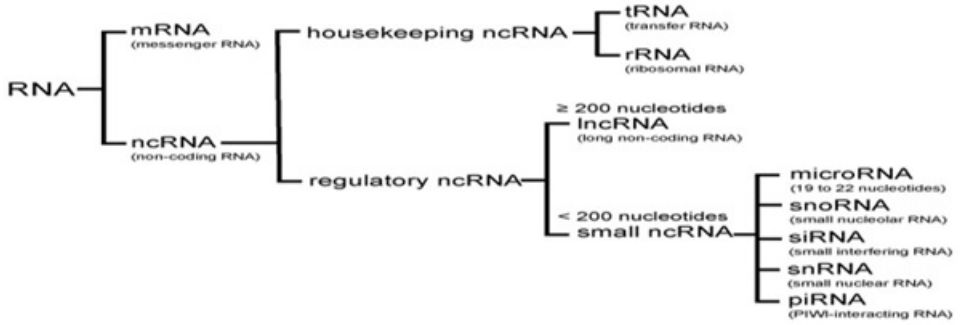

Figure 4: Categories of the non-coding RNAs

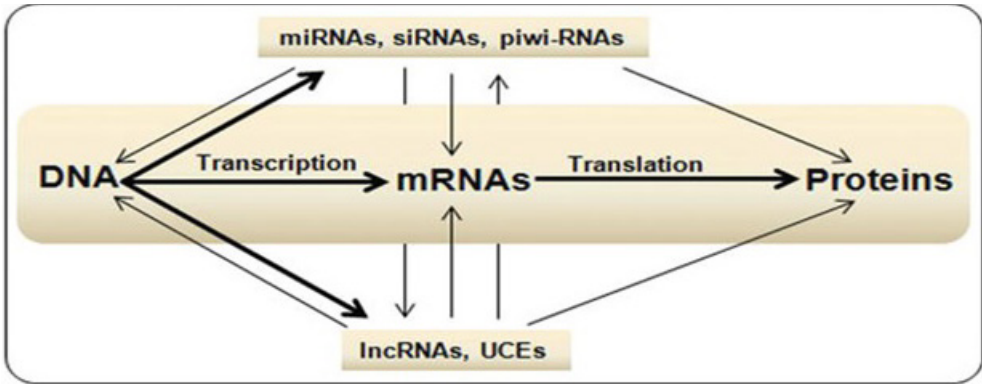

Figure 5: The genetic information from DAN is transcribed (bold arrows) in both coding RNAs (mRNAs) and non-coding RNAs (microRNAs, siRNAs, piwiRNAs, lncRNAs and ECEs), while the mRNA translation in their specific proteins includes complex regulations (regular arrows) performed by all type of ncRNAs.

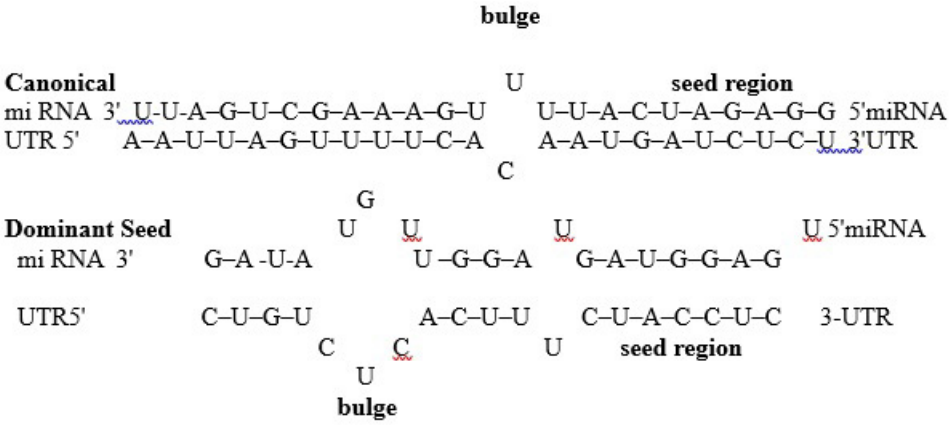

Figure 6: Illustrative secondary structures of the main types of target site duplex. Canonical sites have good or perfect complementarity at both the 5' and 3' ends of the microRNA with a characteristic bulge in the middle

MicroRNA genes are transcribed by enzyme RNA polymerase II that frequently binds to a promoter near DNA sequence that encodes what will be the hairpin loop of the pre-microRNA, resulting in a transcript with modi-fied polyadenylated adenosine nucleotides [poly (A) tail at the 5' end], and spliced animal microRNAs are ini-tially transcribed as part of one arm of ~80 nucleotide RNA stem-loop structure, forming part of several hun-dred nucleotide-long microRNA precursor (pri-microRNA), (Figure 5) (188). RNA polymerase III (Pol III) transcribes microRNAs, particularly those with upstream Alu sequences, transfer RNAs (tRNAs) and the mammalian-wide interspersed repeat (MWIR) promoter units. Dominant seed sites have a perfect 5' seed com-plementarity to the microRNA, but with poor complementarity to the 3' end. Compensatory sites have a mis-match or wobble in the 5' seed region but compensate through excellent complementarity at the 3' end. In the process of Nuclear processing & export, a single pri-microRNA could contain 1-6 microRNA hairpin loop pre-cursors, composed of ~ 70 nucleotide each, flanked by sequences that are needed for processing. The pri-microRNA is longer than the conserved stem-loop structure characteristic of microRNA genes [189].

The double-stranded (ds) RNA hairpin structure is recognized by a nuclear protein known as DiGeorge Syn-drome Critical Region (DGCR), or Pasha in invertebrates [190]. Pre-microRNA spliced directly of introns, by-passing the Microprocessor complex, called "Mitrons" is found in Drosophila, C. elegans and mammals. About 16% pre-microRNAs could be altered by nuclear RNA editing (191). RNA editing could halt nuclear processing and alter cytoplasmic processing and target specificity). Pre-microRNA hairpins are exported from the nucleus to cytoplasm through protein Exportin-5, member of karyopherin family that recognizes a 2-nucleotide overhand left by the RNAase III enzyme Drosha at the 3' end of the pre-microRNA hairpin. The process is energy de-pendent using GTP bound to Ran protein [192].

13.2. Microarrays, Relative Polymerase Chain Reaction (Qpcr) & Digital Pcr for The Absolute Quantification of Micrornas, For The Noninvasive Diagnostic Screening of Colon Cancer in Humans

The discovery of small non-coding protein sequences, 17-27 nucleotides long RNAs (such as microRNAs), has opened new opportunities for a non-invasive test for early diagnosis of many cancers. MicroRNA functions seem to regulate development and apoptosis, and specific microRNAs are critical in oncogenesis, effective in classifying solid and liquid tumors, and serve as oncogenes or suppressor genes [136, 137]. MicroRNA genes are frequently located at fragile sites, as well as minimal regions of loss of heterozygosity, or amplification of common break-point regions, suggesting their involvement in carcinogenesis. Profiles of microRNA expression differ between normal tissues and tumor types, and evidence suggests that microRNA expression profiles can cluster similar tumor types together more accurately than expression profiles of protein-coding mRNA genes. Although exosomal RNA will be missed, a parallel test could be done on microRNAs obtained from stool samples to compare the extent of loss when colonocytes are only used, and an appropriate correction for exsosomal loss can then be made [194]. To ascertain the validity of a microRNA screening test for CC, it must first be validated in a study, using a nested case control epidemiology design and employing a prospective specimen collection, retrospective blind evaluation (PRoBE) of control subjects and test colon cancer patients, as specifically delineated by (NCI's) Early Detection Research Network (EDRN) http://edrn.nci.nih.gov for cancer biomarker discovery studies. Immunoparamagnetic beads can be employed to capture colonocytes from the harsh stool environment, whose extracted fragile total small RNA is stabilized shortly after stool excretion by commercial kits so it does not ever fragment, followed by standardized analytical quantitative microRNA dPCR-chip profiling in

noninvasive stool samples, to develop a panel of few stable microRNAs for absolute quantitative diagnostic screening of early sporadic colon cancer (stage 0-1), more economically and with higher sensitivity and specificity than other CC screening test on the market today [138].

A preliminary global microarray expression analysis study using an exfoliated colonocytes enrichment strategy, which employed control subjects and various stages (0-4) of CC, using Affymetrix GeneChip microRNA 2.0 Array, showed 180 preferentially expressed microRNA genes that were either increased (124 microRNAs), or reduced (56 microRNAs) in expression in stool samples from co -31, -34a, -96, -106a, -133a, -135b, -206, -224 and -302; and 2 Down-Regulated, microRNA-143 and microRNA-145) for further analysis of absolute microRNAs expression by a chip-based digital PCR test, that offers alternate method to qPCR for absolute quantification, by partitioning a sample of DNA or cDNA into many individual, parallel PCR reactions; some of these reactions contain the target molecule (positive), while others do not (negative). A single molecule can be amplified a million-fold or more. During amplification, TaqMan chemistry with dye-labeled probes is used to detect sequence-specific targets. When no target sequence is present, no signal accumulates. Following PCR analysis, the fraction of negative reactions is used to generate an absolute count of the number of target molecules in the sample, without the need for standards or endogenous controls. In conventional qPCR, the signal from wild-type sequences dominates and obscures the signal from rare sequences. By minimizing the effect of competition between targets, dPCR overcomes the difficulties inherent in amplifying rare sequences, and allows for the precise absolute quantification of selected microRNAs [140].

Applied Biosystem QuantStudio™ 3D instrument only performs the imaging and primary analysis of the digital chips. The chips themselves must be cycled offline on a Dual Flat Block GeneAmp® 9700 PCR System or the ProFlex™ 2x Flat PCR System. The QuantStudio™ 3D Digital PCR System can read the digital chip in less than 1 minute, following thermal cycling. The current QuantStudio™ 3D Digital PCR Chip allows for one sample per chip; although, duplexing allows for analysis of two targets per chip. Sample prep for digital PCR is no different than for real-time PCR, when using the QuantStudio™ 3D Digital PCR System. The concentration of cDNA stock can be estimated by including all of the necessary dilution factors into the AnalysisSuite™ software, which gives the copies/µL in the stock. A critical step in dPCR is sample partitioning (i.e., division of each sample into thousands of discrete subunits prior to amplification by PCR, each ideally containing either zero or one (or at most, a few) template molecules. Each partition behaves as an individual PCR reaction –as with real-time PCR—fluorescent FAM probes [or others, as VIC fluorescence]. Samples containing amplified products are considered positive (1, fluorescent), and those without product –with little or no fluorescence (i.e., are negative, 0). The ratio of positives to negatives in each sample is the basis of amplification, unlike real-time qPCR, dPCR does not rely on the number of amplification cycles to determine the initial amount of template nucleic acid in each sample, but it relies on Poisson Statistics to determine the absolute template quantity [146].

It should be noticed that the unique sample partitioning step of dPCR, coupled with Poisson Statistics allows for higher precision than both traditional and qPCR methods; thereby allowing for analysis of rare miRNA targets. The use of a nanofluidic chip provides a convenient mechanism to run thousands of PCR reactions in parallel. Each well is loaded with a mixture of sample, master mix, and Applied Biosystems TaqMan Assay reagents, and individually analyzed to detect the presence (positive) or absence (negative) of an endpoint signal. To account for wells that may have received more than one molecule of the target sequence, a correction factor is applied using the Poisson model. It features a filter set that is optimized for the FAM™, VIC®, and ROX™ dyes, available from Life Technologies [147].

Digital PCR, however, needs a rough estimate of the concentration of microRNAs of interest to be first carried out, in order to make appropriate dilutions; Non-template controls and a RT negative control must be set up for each microRNA, when using a "primer pool method" for retro-transcription; a chip-based dPCR method requires less pipetting steps, which reduces potential PCR contamination, and Quant StudioTM 3D chip has 20,000 fixed reaction wells, which reduces variability of dPCR results.

To avoid bias, and ensure that biomarker selection and outcome assessment will not influence each other, we a prospective specimen collection retrospective blinded evaluation (PRoBE) design randomized selection could be employed. An enrichment and exfoliation strategy of colonocytes from stool for microRNA profiling using Dynal superparamagnetic polystyrene beads coated with a mouse IgG1 monoclonal antibody (Ab) Ber-Ep4, specific for an epitope on the protein moiety of the glycopolypeptide membrane antigen Ep-CAM, which is expressed on the surface of colonocytes and colon carcinoma cells can be used. Comparing the Agilent electrophoretic (18S and 28S) patterns to those obtained from total RNA extracted from whole stool, and differential lysis of colonocytes by RT lysis buffer (Quagen), could be construed as a validation that the electrophoretic pattern observed in stool (18S and 28S) is truly due to the presence of human colonocytes, and not due to stool contamination with Escherichia coli (16S and 23S). While some exsosomal RNA can be released from purified colonocytes into stool, correction for that effect can be made. Hence, for CRC screening, microRNA markers are much more comprehensive and preferable to a DNA-, epigenetic-, mRNA- or a protein-based marker. An added advantage of the use of the stable, nondegradable microRNAs by PCR expression, or chip-based methods is being automatable, which makes them much more eco-

nomical and more easily acceptable by laboratory personnel performing these assays [167].

Stool testing has several advantages over other colon cancer screening methods as it is truly noninvasive and requires no unpleasant cathartic preparation, formal health care visits, or time away from work or routine activities. Unlike sigmoidoscopy, it reflects the full length of the colorectum and samples can be taken in a way that represents the right and left side of the colon. It is believed that colonocytes are released continuously and abundantly into the fecal stream, contrary to blood that is released intermittently as in guaiac FOBT; therefore, this natural enrichment phenomenon partially obviates the need to use a laboratory-enrichment technique to enrich for tumorigenic colonocytes, as for example when blood is used for testing. Furthermore, because testing can be performed on mail-in-specimens, geographic access to stool screening is essentially unimpeded. Results have shown that even the presence of bacterial DNA, non-transformed RNA and other interfering substances in stool does not interfere with measuring microRNA expression, when an enrichment method such as the immunological paramagnetic capture method is used, and when appropriate PCR primers are employed. Besides, stool colonocytes contain much more miRNA than that available in free circulation such as in plasma.

Routine extraction of total small RNA from Stool Samples can be carried out using commercial RNA extraction preparations, which provide the advantage of manufacturer's established validation and quality control standards, increasing the probability of good results to extract high quality total RNA from an environment as hostile as stool; thus, shattering the myth that it is difficult to employ RNA as a screening substrate, as fragmented RNA results in poor cDNA synthesis and ultimately in less than optimal PCR amplification. This step is followed by reverse Transcription (RT) and preparation of single stranded copy deoxy ribonucleic acid (ss-cDNA) [169].

Compared to real-time quantitative PCR (qPCR), dPCR clearly offers more sensitive and considerably more reproducible clinical methods that could lend themselves to diagnostic, prognostic, and predictive tests. But for this to be realized, the technology will need to be further developed to reduce cost and simplify application. Concomitantly the preclinical research will need be reported with a comprehensive understanding of the associated errors. The term "absolute quantification" used in dPCR refers to an estimate derived from the count of the proportion of positive partitions relative to the total number of partitions and their known volume. When the sample is sufficiently dilute, most partitions will not contain template and those that do are most likely to contain single molecules. As the sample becomes more concentrated, the chance of more than 1 molecule being present within a positive partition increase. This does not pose too great of a challenge, because the distribution of molecules throughout the partitions approximates a Poisson distribution, and a Poisson correction is applied. The dynamic range of a dPCR assay can extend beyond the number of partitions analyzed but the assay precision deteriorates at each end. In contrast, qPCR precision deteriorates only at low copy numbers [165].

The dPCR method benefits from a far more predictable variance than the qPCR, but dPCR is susceptible to upstream errors associated with factors like sampling and extraction. dPCR can also suffer systematic bias, particularly leading to underestimation, and internal positive controls are likely to be as important for dPCR as they are for qPCR, especially when reporting the absence of a sequence. Calibration curves are frequently employed to reduce the error associated with qPCR, but they in turn are challenging to select, assign value, and apply in a manner that will be reproducible; their application also contains inherent error that is almost never considered [167].

A key problem with applying qPCR to areas such as the discovery of biomarkers that will eventually be translated to clinical care, is understanding whether poor reproducibility is biological, or if it is due to issues related the fact the qPCR technique is difficult to perform reproducibly. Implement dMIQUE Guidelines on dPCR data analysis helps to standardize experimental protocol, maximize inefficient utilization of resources, and enhance the impact of this technology. To access test performance characteristics (PCT) of the microRNA approach, the copies/µl values of the microRNA gene panel (or a derived miRNA index, IMP) obtained from stool/colonelcy samples of normal subjects and colon cancer patients with high sensitivity and specificity are compared to the commonly used guardian FONT test and with endoscopy results obtained from patients' medical records to access TPC of the miRNA approach. False positive discovery rates (expected proportion of incorrect assignment among the accepted assignments) will be assessed in our proposed approach by statistical methods, as it could reflect on the cost effectiveness of our test. The number of optimal microRNA genes (whether 14 or less) to achieve an optimum gene panel can be established by statistics. Statistical methods, both parametric and non-parametric methods can be used for validating the microRNA approach. Cross-validation can be used to protect against over-fitting. Bioinformatics methods can be used to correlate seed microRNA with target mRNA data [168, 169].

Providing numerical underpinning of dPCR as a function of total small RNA can be ascertained by use of cytological methods on purified colonocytes employing Papanicolaou and Giemsa staining. A known number of the colonocytes isolated from 1g stool (from normal and neoplastic preparations), extracting total RNA from them to determine the actual amount of total RNA per stool sample, and determining the average copies/µl value from the panel of selected microRNAs from dPCR using the QuantStudioTM 3D Digital PCR Chip instrument, will ultimately give an average value per a certain amount (pg or ng) of total RNA [169].

To measure the clinical utility of gene expression testing as a screening test, the diagnostic sensitivity and specificity of the microRNA gene panel selected, or a derived PMI, can be compared to the published sensitivity and specificity of the commonly employed diagnostic screening test, guaiac FOBT, which for over 3 decades in large adenoma averaged < 12%, and in carcinoma averaged ~ 30%, and the specificity averaged ~ 95%, and to the gold screening standard colonoscopy results obtained from participants' medical records that averaged 87% for sensitivity and 100% for specificity. The limitations of FOBT are biologically inescapable and cannot be reversed by technological advances. On the other hand, based on our data, if our hypothesis proves valid, we can screen colon cancer, particularly at the per-malignant stage, with > 90% sensitivity and > 95% specificity, employing 15 microRNA genes in a functional assay, which is better than any available non-invasive test. Thus, a large number of patients will be spared the discomfort, risk and expense of screening colonoscopy. Only those patients truly at risk of having a colon cancer will need to undergo colonoscopy [136-140, 146, 167, 169].

Using the copies/ul results from the panel of genes selected obtained from stool samples of normal, and from stool samples of cancer patients, a 2 x 2 tables can be used to predict a microRNA index to determine the clinical sensitivity and specificity of the microRNA assay from mRNAs stool specimens' results. dPCR is the most practical, least labor-intensive and economical approach to quantify microRNA as a noninvasive diagnostic test to screen for CC, using automatic RNA extraction technology. Other alternate methods such as use of Real-Time qPCR, Next Generation Sequencing (NGS) technologies, plate assay technology to study microRNA expression, and microRNA measurements from exosomes and microvessicles extracted from stool, can provide alternate standardized technical methods for achieving microRNA quantification, although these methods will not be as precise, nor as economical as using dPCR technology [169].

14. MicroRNA Targets & Their Prediction

Plant microRNAs pair perfectly with their mRNA targets, resulting in gene repression by cleavage of target transcripts [195]. On the other hand, animal microRNAs recognize their target mRNA by using a 6-8 nucleo-tides, referred to as the "seed region" located at the 5' end of the microRNA, which results in little pairing, not sufficient to lead to target mRNA's cleavage [196]. In animals, a single microRNA could have hundreds of dif-ferent microRNA targets, and each target could be regulated by several microRNAs. Mammalian microRNAs could have many unique conserved targets, in the hundreds [197], and studies have shown that a single mi-croRNA species could repress the production of hundreds of proteins, in imperfect complementarity, although often the repression is much less than 2-fold [198].

Perfect complementarity leads to target mRNA degradation [199]. Moreover, microRNA was shown to function as ligand to activate signaling pathways in tumor cells that secreted microRNA-21/microR-29a that directly bind to murine Toll-like receptor 7, or human Toll like receptor 8 transferred into immune cells, resulting in a Toll-like receptor-mediated prometastatic inflammatory response, which may ultimately lead to tumor growth and metastasis. Additionally, microRNA was shown to affect nuclear factor KB signaling pathway in natural killer cells through direct interaction with ligand Toll-like receptor [200].

Several computational methods to identify target genes that rely on the conservation of binding sites (e.g., Target Scan) [201, 202], while others rely on site accessibility and thermodynamic properties to filter the seed binding sites (e.g. miRanda) [203]. Prediction algorithms use a combination of different features to increase their accuracy, and to compensate for the limitations of the individual features. Machine learning based algo-rithms rely on parametrization of biological data and other predicted features in genomics to generate more ac-curately validated microRNA-target interaction (e.g., TarpmiR, miRGen++, MBSTAR) [204, 205]. Based on prediction accuracy algorithm and the fact that most of the prediction databases were not updated for some years, miRWalk learning-based approach was employed to increase the prediction accuracy and sensitivity [206-298].

There are common features and less common features of microRNA Target Prediction tools. Four commonly used features for microRNA target prediction tools are: a) seed match, b) conservation, c) free energy, and d) site accessibility. The seed sequence of a microRNA is defined as the first 2–8 nucleotides starting at the 5′ end and counting toward the 3′ end [202] (Figure 6).

MicroRNA position number is shown in blue. The seed sequence refers to nucleotides in microRNA position numbers 2–8. Flank refers to the mRNA sequence on either side of the region corresponding to the miRNA seed sequence. WC matches in the seed sequence are shown in red, and an example of G-U wobble in the seed se-quence is shown in green. There are several types of seed matches depending on the algorithm: i) 6mer: A per-fect WC match between the microRNA seed and mRNA for six nucleotides; ii) 7mer-m8: A perfect WC match from nucleotides 2–8 of the microRNA seed; iii) 7mer-A1: A perfect WC match from nucleotides 2–7 of the microRNA seed in addition to an A across from the microRNA nucleotide 1, and iv) 8mer: A perfect WC match from nucleotides 2–8 of the microRNA seed in addition to an A across from the microRNA nucleotide. Adapted from open source reference [202].

Conservation refers to the maintenance of a sequence across species. Conservation analysis focuses on regions in the 3′ UTR, the 5′ UTR, the microRNA, or any combination of the three. There is generally a higher conserva-tion in the microRNA seed region than in the non-seed region [196]. In a small proportion of the

microRNA: mRNA target interactions, there is conserved pairing at the 3′ end of the microRNA, which can compensate for seed mismatches, and these sites are called 3′ compensatory sites [197]. Conservation analysis has been applied to the promoter regions of microRNAs and their target genes [202]. Gibbs free energy is considered to be a measure of the stability of a biological system. If the binding of a microRNA to a candidate target mRNA is predicted to be stable, it is considered more likely to be a true target of the miRNA. Because of the difficulty in measuring free energy directly, a change in free energy during a reaction (ΔG) is often considered. Reactions with a negative ΔG have less energy available to react in the future, resulting in systems with increased stability. By predicting how the microRNA and its candidate target hybridize, regions of high and low free energy can be inferred, and the overall ΔG can be used as an indicator of the strength of bounding (Figure 7) [203].

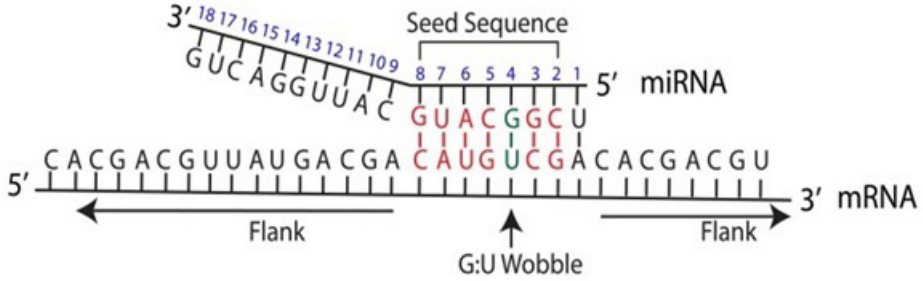

Figure 6: microRNA: mRNA target interaction. A schematic of microRNA interaction with its mRNA target

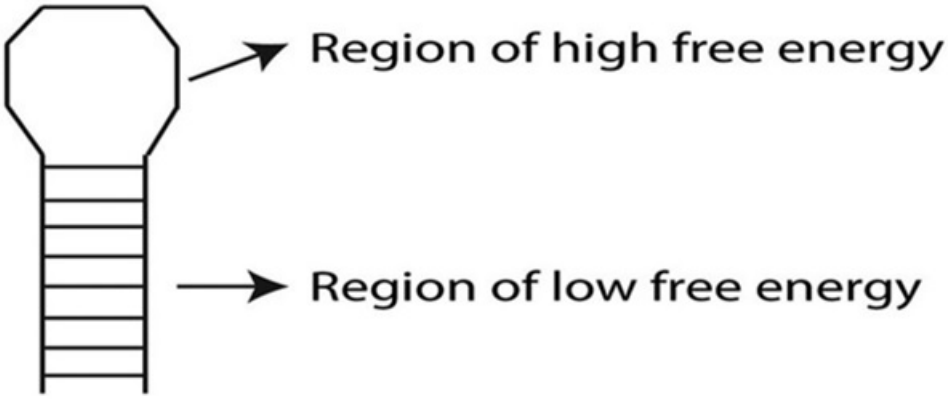

Figure 7: Schematic overview of free energy (ΔG) analysis of predicted RNA hybridization structure, showing a hairpin loop that corresponds to a region of high free energy (a + ve ΔG), and the stem corresponding to a region of low free energy (a - ve ΔG))

Site accessibility is a measure of the ease with which a microRNA can locate and hybridize with an mRNA target. Following transcription, mRNA assumes a secondary structure, which can interfere with a microRNA's ability to bind to a target site. MicroRNA: mRNA hybridization involves a two-step process in which a mi-croRNA binds first to a short accessible region of the mRNA. The mRNA secondary structure then unfolds as the microRNA completes binding to a target [204]. Therefore, to assess whether or not a mRNA is the target of a certain microRNA, the predicted amount of energy required to make a site accessible to a microRNA is evaluated. A fewer common features in characterizing microRNA: mRNA target interactions are target-site abun-dance, which is a measure of how many target sites occur in a 3′ UTR [205] Local AU content refers to the concentration of A and U nucleotides flanking the corresponding seed region of the microRNA [206]. GU wobble in the seed match refers to the allowance of a G pairing with a U instead of a C. 3′ compensatory pair-ing refers to base pair matching with microRNA nucleotides 12–17. Seed pairing stability is the calculated free energy of the predicted duplex [207]. Position contribution analyzes the position of the target site within the mRNA [208]. A machine-learning approach uses training data to develop a model of microRNA targets, and then uses the model as part of the microRNA-prediction process. Machine-learning techniques are likely to use more features in their predictions as they can be trained to determine the predictive power of each feature on positive and negative data sets [209].

Several computational target prediction tools have been developed that apply various distinct and overlapping algorithms, which are continually being modified as more targets are validated, and can distinguish three types of target sites: 5’-dominant canonical,5’-dominant seed only and 3’-compensatory [210-212]. They differ by the level of complementarity of microRNA sequences to the site sequences. The main approach looks for sequence complementarity and/or for favorable microRNA-target duplex thermodynamics [213]. In order to increase the signal to noise ratio, some methods require strict complementarity between the seed

region of the microRNA and any predicted target. Conservation of binding sites is also often used as a metric to improve the raw results. Evidence suggests that the position of a binding site in the 3'UTR may be important along with its context in terms of the secondary structure of the target molecule. Some of the newer approaches utilize this information in order to make more informed target predictions. Prediction algorithms used by the most widely utilized engines rely generally on base pairing between the seed sequence of the microRNA and the 3′-UTR of its target, in addi-tion to evolutionary conservation of the targeted sequence. While "most functional mRNA-microRNA pairing resides in the 3′-UTRs," microRNA target sites are also found within the coding region of a gene, albeit, at a much lower frequency [214].

Recently, a large-scale RNA sequencing study was carried out to experimentally identify genes that are downregulated by 25 microRNAs. This RNA-seq dataset is combined with public microRNA target binding data to systematically identify microRNA targeting features that are characteristic of both microRNA binding and target downregulation. By integrating these common features in a machine learning framework, we a vali-date and improved computational model for genome-wide microRNA target prediction. It should be noted that no conserved microRNAs-targeted sites respond equally well to inhibition by microRNAs. However, these no conserved sites appear to be present in genes that are not expressed in the same tissue as the targeting mi-croRNAs. On the other hand, genes that preferentially coexist with microRNAs in a specific tissue are thought to have evolved through selective elimination of the targeting sites and are known as "antitargets". Hence, there are also target prediction applications that do not rely on target site conservation, such as MicroTar, which may be useful in some instances [215].

It is noted that while the three target prediction applications described above overlap in many predicted tar-gets, they diverge in others. Thus, it might be beneficial to search all the databases for potential targets of a microRNA of interest. However, one needs to consider that not all predicted targets are genuine, as they may be subject to spatial or temporal restrictions. In addition, binding to the targeting site might be modulated by 3′-UTR cis-acting sequences or transacting factors. Moreover, a single 3′-UTR may be targeted by multiple mi-croRNA. Thus, the level of a mRNA or its translation product is governed by the combinational effect of its tar-geting microRNA [216].

The complementarity between microRNA and target could have been seen as advantageous for computational analysis, although other features of microRNA-UTRs association complicate the situation. The usual sequence alignment algorithms assume longer sequences than the prediction of microRNA targets of ~ 20-23nt of mi-croRNAs. This short length makes ranking and scoring of targets difficult, as statistical techniques for sequence matching such as Karlin-Altschul statistics require longer sequences [215]. Binding sites actually consist of regions of complementarity, bulges and mismatches [216]. As standard sequence analysis tools were designed for sequences with longer stretches of matches and less gaps, they are much less useful for microRNA target prediction. It is now understood that position 2 to 7 of microRNAs, the so called 'seed' region, have been described as one of the key specificity determinants of binding and requires perfect complementarity [210, 211].

Unfortunately, the chance of finding a match to an arbitrary 7nt sequence in any given 3'UTR is quite high. Additionally, effective regulation of transcript translation requires that microRNAs and their targets are located in the same cellular compartments. Knowledge of expression localization of microRNAs and their targets would hence be useful for narrowing the scope of microRNA seed searches [217].

15. Improving Microrna Research Tools and Reagents

Because of the diversity of microRNA expression patterns that can be associated with several cancerous and noncancerous diseases, the ability of technologies to detect different patterns of microRNA expression in sam-ples of limited size is becoming quite important. In addition, as with other diseases where expression patterns of markers may vary between individuals, the ability to analyze expression pattern differences between different individuals, for research and diagnostics purposes, requires the use of new assay technologies and bioinformatics analysis tools. In both areas, multiplexing assay platforms and new data analysis tools are providing solutions to these challenges [218].

In order to gain insight into these tiny regulators, researchers around the world are asking fundamental re-search questions such as: What microRNAs are expressed? Where and when are microRNAs being expressed, what cell processes do microRNAs regulate? and What genes do these microRNAs control?

As many microRNA sequences are cataloged in the miRBase Sequence Database. Release 22 of the database contains 38589 entries representing hairpin precursor microRNAs expressing 48885 mature RNA products in 271 species. The data are freely available through the web interface at http://www.mirbase.org/, and in flat file form ftp://mirbase.org/pub/mirbase/.f10031. 10031 new hairpin sequences and 13149 new mature products have been added, and increase in the number of sequences of over a third [219].

The first interpretation of the term "liquid biopsy" in cancer originated in 2010 when circulating tumor cells (CTC) were proposed as an alternative to conventional breast cancer biopsy for prognosis and evaluation of treatment responses [217-219]. Subsequently, clinical applications of liquid biopsies have diversified from de-tecting early-stage cancer to monitoring tumor progression, assessing tumor heterogeneity and residual disease, and potentially monitoring therapeutic response to various surgical and chemotherapeutic interventions [218]. In this context. Several companies

are developing drugs targeting microiRNAs for cardiovascular diseases, some infectious diseases, metabolic diseases, cancer, and some other noncancerous diseases. Because microRNAs are involved with a broad range of human diseases, the development of new effective microRNA targeted treat-ment strategies has a bright future. (Table 2) shows some of the microRNAs that are involved in cancer. Although the information about microRNA biology has grown, we still do not completely understand the mechanism of microRNA gene regulation

Table 2. Available DNA sequencing technologies

Technology*	Approach	Read length	bp/run	Run time	Company/web	Reference
Automated Sanger sequencer 96 capillary array ABI3730xI	*In vivo* synthesis in the presence of dye terminator	From700 to 900 bp	96 kb	3h	Applied Biosytem www. appliedbyosystems.com	142
454/Roche FLX system	Pyrosequencing on solid support	200–300 bp	80–120 Mb	4h	Roche Applied Science www.roche-applied-science.com	144
Illumina/Solexa	Sequencing by synthesis of single molecule arrays with reversible terminators	30–40 bp	1 Gb	2-3h	Illumina, Inc http://www.illumina.com/	145
ABI/SOLiD	Massively parallel sequencing by ligation-hybridization	35 bp	1–3 Gb	8d	Applied Biosytem www. appliedbyosystems.com	146

Table 2: Comparison of different microRNAs detection methods

Method	Advantage	Disadvantage	RNA Input
In situ hybridization	Preserves tissue architecture Multiplexed Quantitative	Labor intensive Relatively expensive New method	N/A
Nanostring expression	Limited profiling Multiplexed Quantitative High sensitivity & Specificity	Relatively expensive Emerging procedure Relative quantification	ng- ng-ug
MicroRNA Microarray	Clinical traceability	Low sensitivity & specificity	ng-ug
Hybridization based detection bioinformatics analysis	Comprehensive profiling Multiplexed	Relatively expensive & requires	ng-ug
RT-qPCR Relative Poly- merase Amplification based detection	Clinical tractability Limited Profiling multiplexed , Quantitative high sensitivity&specificity Relatively inexpensive	Relative quantification Require a Normalizer ensitive to contaminants Needs statistical analysis	<ng
Absolute digital dPCR Quantifixation	Clinical tractability Multiplexed, quantitative Limited profiling Does not require a normalizer Multiplexed, quantitative High sensitivity & specificity Relatively inexpensive	Absolute quantification Sensitive to contaminants Needs a sophisticated sophisticated statistical analysis	ng-ug
Sequencing-based (NGS) detection	Comprehensive profiling Multiplexed, quantitative High sensitivity & specificity Nearly error-free detection)	Relatively labor intensive. has a 3'adapter ligation bias Relatively more expensive & Requires bioinformatics analysis	ng-ug

Table 2: MicroRNAs involved in cancer

microRNA	**Genomic Location**	**Expression in patients**	**Deregulation Mechanism**	**Function**	**Targets**	**Experimental data**	**Therapeutic Strategy**
miRNA-15a/ miRNA-16-1	13q31	Down in CLL, prostate Cancer and pituitary adenomas	Genomic loss Mutations Positive reg. by p53	TS	*BCL-2 MCMCL-1*	In vitro over-expression induces apoptosis in CLL and prostate cancer cells In vivo silencing causes CLL in mice	Mimics Vector-based (viral) Drugs

Let-7a-2	11q24	Down in lung, co-lon breast, ovarian and stomach cancer	Negative reg. by *MY*	TS	*K-RAS, N-RAS CDK6, CDC25A HMGA2 MYC*	In vitro over-expression reduces cell growth in lung, breast and colon cancer cells In vivo over-expression reduces breast & lung tumor burden in mice	Mimics Vector-based (viral) Drugs
miRNA-29b-1/ miRNA-29a miRNA-29b-2/ miRNA-29c	7q32 1q30	Down in NPM1 wt AML, CLL, Lung and breast cancer, cholangiocarcino-ma, lymphoma, hepatocarcinoma & and rhabdomyosar-coma	Gnomic loss Negative reg. by MY Positive reg. by p53	TS	*ML-1, CDK6 CL-1, DNMT1 DNMT3a and b*	In vitro over expres-sion induces apopto-sis, inhibits cell pro-liferation and induces DNA hypomethylation in several cancers In vivo over-expression inhibits tumorigenicity in AML, liver and lung cancer	Mimics Vector-based (viral) Drugs
miRNA-34a miRNA-34b and c	1p36 11q23	Down in colon, lung, breast, kid-ney, bladder cancer and melanoma cell lines Down in neuro-blastoma	Methylation reg. Positive reg. by p53 Deletion	TS	*CDK4, CDK6, CCNE2,-D1 MET, MYC, CREB,E2F3,BCl-21*	In vitro over-expression induce cell cycle arrest apoptosis and inhibits cell proliferation	Mimics Vector-based (viral) Drugs
miRNA-26a	3p22	Down in Liver cancer	Negative reg. by *MYC*	TS	*CCND2, CCNE2*	Restoration of miR-NA-26 inhibits MYC-induced liver cancer	Vector-based (viral)
miRNA-155	21q21	Up in high risk CLL, AML Lung, colon, breast cancer and lymphomas	Positive reg. by NFκB118	OG	SHIP-1 and CEBPb	Over-expression in HSC induce myeloid proliferation and block erythropoiesis in mice In vivo over-expression in lym-phocytes induce pre-B lym-phoma/Leukemia	Antisense oligos miR-MASK Sponges, Drugs
miRNA-17-92	13q22	Up in lung, breast, colon, and stomach cancer, myeloma and t(11q23) AML	Amplification Transcription (E2F and MYC)	OG	ccBIM, PTEN CDKN1A	Cooperate with C-MYC to induce lym-phoma In vivo over-expression in lym-phocytes induce lymphoid prolifera-tion and autoimmun-ity	Antisense oligos miR-MASK Sponges, Drugs

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