Circulating miRNAs a diagnostic tool for cancer detection?

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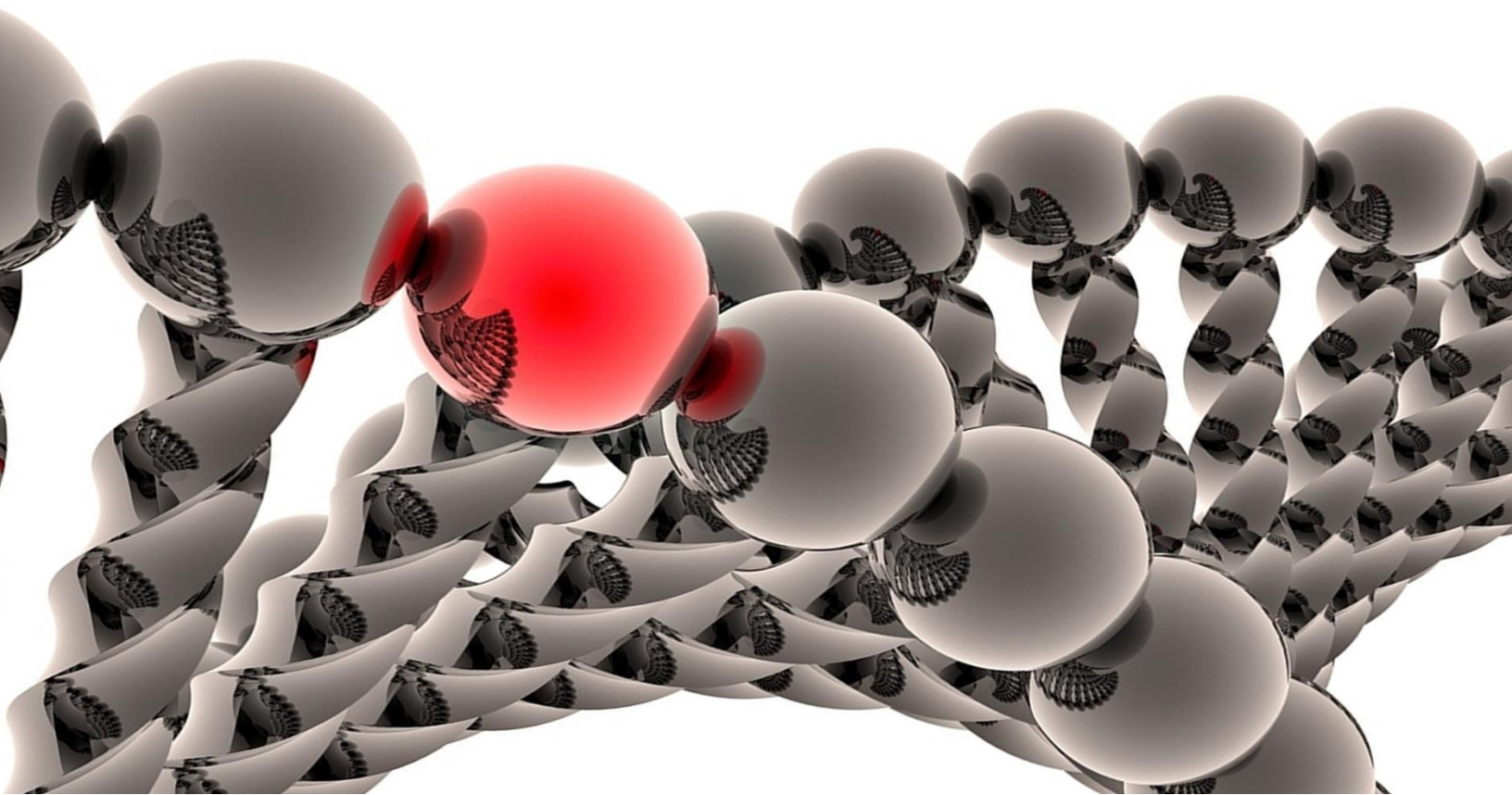

Abstract

MicroRNAs are small non-coding RNA sequences that can inhibit mRNA at post-transcriptional level. Many biological processes are influenced by microRNAs, for example: differentiation, proliferation, development and apoptosis. Deregulation in miRNA expression affects the protein expression and therefore it can cause all different kinds of diseases. The possibility to isolate miRNAs out of body fluids raised the question if circulating miRNAs could be used as biomarker for diseases. This review will discuss the possibility to use circulating miRNAs as diagnostic tool for cancer detection and in particularly lung cancer. In blood miRNAs can be found in serum and plasma as cell-free miRNAs and in extracellular vesicles. Alterations in specific miRNA levels are associated with specific cancers. Suggested was that the altered levels can be used for the diagnosis of cancer. But before miRNAs can be used as diagnostic tool a reliable and reproducible test is required. In technical point of view there are still problems. Pre-analytical variables can cause alterations in miRNA levels but also the use of different extraction methods can influence the miRNA level. For lung cancer specific miRNAs are found for detection and subclassification. Besides the detection, circulating miRNAs are also associated with the size and advanced stage and may have a prognostic value as well. Furthermore miRNAs are associated with different drug resistances and for that may have influence on the therapy strategy. The conclusion is that miRNAs could give a lot of information about a tumor for diagnosis, prognosis and therapy strategy. But still research needs to be done to create a reliable and reproducible test.

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Introduction miRNAs

The *lin-4* gene was known for controlling development in *Caenorhabditis elegans*. Lin-4 acts by negatively regulating the level of the LIN-14 protein[17]. In 1993 Ambros et al. discovered that the *lin-4* gene produces a small pair of non-coding RNAs instead of producing a protein. The small pair of non-coding RNAs produced by *lin-4* was the first discovered microRNA (miRNA)[1]. The *lin-4* gene was for a long time the only known microRNA. This all changed upon the discovery in 2000 that the *let-7* gene also encodes for a small regulatory non-coding RNA[18]. In the same year homologs of the *let-7* gene were identified in the other genomes, including in humans[40]. Today there are in humans 1881 miRNAs known[41]. MicroRNA are approximately 22 nucleotide long non-coding RNA sequences and play a role in the post-transcriptional regulation of gene expression[2].

Biosynthesis

Genes coding for miRNAs can be found as clusters or as distinct miRNAs. miRNA transcripts can be transcribed from introns within genes as well. Transcriptions of the miRNA genes can be done by two polymerases, pol II and pol III. The Intron-encoded miRNAs are the most common variant and can only be transcribed by pol II[1,3,4]. The first product formed after transcription is the primary (pri)-miRNA molecule. This is a several hundred nucleotides long molecule with on both 5' and 3' sides of the miRNA an extended sequence. Drosha crops the pri-miRNA into a shorter hairpin-shaped pre-miRNA. Exportin-5 exports the pre-miRNA towards the cytoplasm. Dicer carries out the cleaving of the pre-miRNA into the mature miRNA duplex. One strand of the duplex is degraded by an unknown nuclease and the other strand becomes the mature miRNA[4].

Cellular Functions

The mature miRNA is incorporated in a RNA-induced silencing complex (RISC). The RISC complex interacts with its messenger RNA (mRNA) targeted through (partial) complementary sequences, which are then posttranscriptionally silenced[2]. There are two mechanisms in which RISC can silence the mRNA, by translational repression or degradation of the mRNA (Fig. 1) [7,8]. Degradation is initiated by RISC through deadenylation of the mRNA's poly A tail[9]. MicroRNAs are involved in many biological processes, for example: differentiation, proliferation, development and apoptosis[5]. Due to the partial complementary sequence one miRNA is able to target multiple mRNAs.

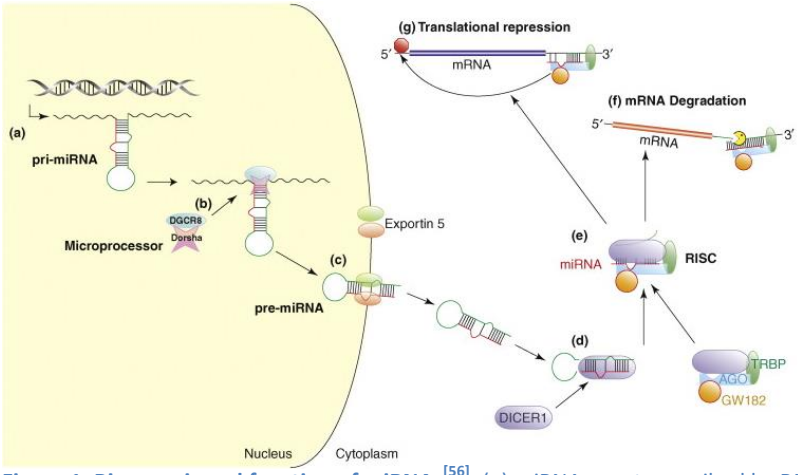

Figure 1: Biogenesis and function of miRNAs[56]: (**a**) miRNAs are transcribed by RNA polymerase II & III. (**b**) Drosha cleaves the pre-miRNA from the pri-miRNA. (**c**) Exportin-5 transports pre-miRNA towards the cytoplasm. (**d**) Dicer cleaves the pre-miRNA and one mature stand remains. (**e**) RISC complex with the mature miRNA strand. (**f**) RISC induces mRNA degradation. (**g**) RISC induces translational repression.

miRNA and Cancer

MicroRNAs can be functioning as oncogenes and tumor suppressor genes depending on their posttranscriptional regulation target. The first evidence for this was a deletion on 13q14 associated with chronic lymphocitic leukemia(CCL), multiple myeloma and prostate cancer. The loss of a tumor suppressor gene was the most likely explanation, but instead of a tumor suppressor gene two miRNA genes were found at this locus, namely miR-15 and miR-16. A study showed that the majority of the CCL cases are caused by deletion or down-regulation of these two miRNAs[10]. Genetic alterations can cause an aberrant microRNA expression profile. These aberrant expression profiles can characterize different tumors. Thus, miRNAs may be used as possible biomarkers. This review will discuss the possibility to use circulating miRNAs as diagnostic tool for cancer detection and in particularly lung cancer.

Expression profiles of tumor cells

An accurate diagnosis is vital for the treatment of patients with cancer. For tumor classification there are multiple techniques, for example immunohistochemistry or detection of biomarkers in blood. Another diagnostic technique to classify tumors is by the gene expression profiles. A study was preformed to investigate 16.063 mRNA expressions by using a microarray. The cohort exists out of 218 tumor samples from 14 different tumor types and 90 normal tissue samples. Results showed that 78% of the tumors could be accurate determined with the mRNA expression profiles[11]. Besides this study a lot of other studies showed the possibility to use mRNA profiling for classification, for example in colon cancer[59] and breast cancer[60]. But since the discovery of miRNAs and the understanding of its mechanism it has been suggested that miRNAs profiling is a better diagnostic tool then mRNA profiling. Reasons for this assumption is that in contrast to miRNAs only a small part of the mRNAs have a regulatory function[12]. And miRNAs are also more stable then mRNAs[13]. This would mean that miRNAs are superior to mRNAs because they can more about the tumor and are more suitable for laboratory use.

Lu et al showed first the possibility that miRNAs could be informative biomarkers. Analysis of 217 miRNAs in 334 samples showed that tumor cells and normal cells differ in miRNA expression profiles. And more interesting was that miRNA were able to distinguish different tumors based on their expression profiles. From the 217 microRNAs several were higher expressed or stayed the same, but 129 miRNAs had a lower expression compared with the normal tissue. Even poorly differentiated tumor were able to be classified successfully[14]. Whereas the mRNA expression profiling wasn't able to classify the poorly differentiated tumors at all[11]. Another study confirmed these findings by using 228 miRNA expression profiles. The expression profiles were gained from 540 samples consisting of 363 tumor samples (six different tumor types) and 177 normal tissue samples. Their findings concluded as well that there was a good distinction between expression profiles for different tissues and cancers[15].

miRNAs as circulating biomarkers

Diagnosis based on expression profiles in tumor cells require invasive techniques to obtain the tissue samples. A less invasive method to establish a diagnosis is the detection of biomarkers in blood or other body fluids. Most common used biomarkers are proteins and antibodies in blood, for example: aminotransferase (AST) for liver functions, and prostate specific antigen (PSA) for prostate cancer[16]. With the knowledge that miRNAs are tissue specific and are deregulated in cancers, speculations were made if miRNAs in blood could be used as biomarker. Mitchell et al. proved that miRNAs were circulating in blood. Their results showed also that they were able to distinguish healthy mice from mice with prostate cancer by elevated levels of miR-141. In addition, results showed that miRNAs were even very stable in blood[19]. Besides the discovery of miRNAs in blood other studies showed the possibility to extract miRNAs from: urine[20], feces[21], sputum[22] and Cerebrospinal fluid[61].

miRNA as biomarker in serum or plasma

This review will only discuss the possibility to detect miRNAs in blood. A possibility is to isolate miRNAs from plasma or serum. Circulating miRNAs in blood are results of apoptotic and necrotic cell death[82]. Although active secretion has been suggested as possible source for circulating miRNAs as well[83]. In blood the stability of the miRNA seems to be very stable. After 24 hours of incubation at room temperature the concentration of miRNAs remains the same. Also after 8 freeze thaw cycles the miRNA concentration was unchanged[19]. This stability is caused by natural complex forming with Ago proteins, in particularly Ago2. Ago proteins are part of the RISC complex. The Ago2/miRNA complex is nuclease and protease resistant. Studies reported that >90% of all circulating miRNAs are vesicle free miRNAs[24,25]. Increased or decreased levels of miRNAs in plasma and serum have already been associated with different diseases. For example plasma levels of miR-34 were elevated in oral squamous cell carcinoma patients[23]. The levels of miR-21 and miR-184 are elevated in other squamous cell carcinomas[30,31]. In colorectal cancer miR-29a and miR-92a are both found to be increased in plasma[32]. In serum increased levels of miR-21, -92, -93, -126 and -29a are associated with ovarian cancer, but also decreased levels of miR-155, -127 and -99b[33].

Extracellular Vesicles

Exosomes and microvesicles belong both to the extracellular vesicles. Both vesicles are released by healthy and damaged cells. Exosomes are formed in multivesicular endosomes (MVE) and are released after fusion the plasma membrane, the diameter of an exosome is 40-100nm. Microvesicles are originated from budding with the plasma membrane and have a diameter of 100nm to 1µm[26]. Extracellular vesicles can contain miRNA, proteins and mRNAs. Living cells secrete EV's but the function of the extracellular vesicles remains not entirely understood, although there is evidence that they act as mediators for cell-cell communication[27,28,29]. Extracellular vesicles are another source of circulating miRNAs in blood. Different diseases are characterized by an aberrant miRNA level in EV's. Elevated levels of miRNAs in EV's, including miR-200c and miR-214, are associated with ovarian cancer[34]. Increased levels of miR-196 in EV's is suggested as marker for glioblastoma[35]. Ovarian cancer patients showed also elevated levels of miR-200c in plasma[58], but in plasma or serum miR-214 or -196 are not reported with altered levels. Thus, there may be a difference between cell-free circulating miRNAs and miRNAs extracted from EV's.

Pre-analytical variables

Circulating miRNAs have been proposed as biomarker for diagnostic or even prognostic purposes. But before circulating miRNAs can be used as diagnostic tool, it is necessary to create a reliable and reproducible test. The influence of pre-analytical variables during sample handling can cause changes in the miRNA profile, for example: hemolysis or co-purified inhibitors. Another variable is the individual variance which is probably the least standardizable. The variables can cause incorrect profiles and thereby a wrong diagnosis.

Hemolysis during sample preparation will release the miRNAs of red blood cells into the plasma. Pritchard et al. demonstrated that miR-92a was increased by hemolysis[36]. Increased levels of miR-92a were also associated with colorectal cancer[32]. Thus, increased levels of miR-92 may result in a wrong diagnosis because of improper handling during isolation. Polymerase inhibitors co-purified with the miRNAs can influence the detection as well. The polymerase inhibitors can inhibit taq polymerase or reverse transcriptase which may be needed for further analysis of the miRNAs[37].

Individual variance can lead to variation in miRNA expression and have to be taken into account such as: diet, exercise or environmental chemicals. Vitamins can alter the miRNA expression profiles. Peng et al. showed that miR-182 profile was altered when breast epithelial cells were exposed to stress, serum starvation. But in the presence of 25-hydroxyvitamin D, a major vitamin D metabolite, alterations were inhibited[38]. Exercise can also influence circulating miRNA levels. Changes in 34 circulating miRNAs were observed after performing exercises[39].

miRNA extraction methods

Plasma and serum are both used to isolate miRNAs. The difference between plasma and serum is the absence of clotting factors in serum. Higher concentrations of miRNAs are found in sera[42]. Suggestions were made that these different concentrations were caused through the release of miRNAs by platelets and other blood cells during the clotting process. Willeit et al. investigated the influence of released miRNAs upon platelet activation. Different miRNA levels were found in serum compared with plasma. The miRs-126, -150, -191 and -223 were found at higher concentrations in serum as in plasma[43]. Anticoagulants may affect the results as well. EDTA, citrate and heparin are well known anticoagulants. EDTA is the preferred above citrate and heparin, result of their possible inhibitory effect on PCR techniques[44,45].

Different extraction methods are used to extract miRNAs out of blood. Phenol Chloroform is a common used method for miRNA extraction, mostly marketed as Trizol. Other commercial kits often uses columns to isolate miRNAs. Multiple studies have reported different miRNA expressions when using different extraction methods. Mcdonald et al. compared four different commercial kits: mirVana PARIS kit, miRNeasy Mini Kit, mirPremier microRNA Isolation Kit and High Pure miRNA Isolation Kit. Their data showed that mirVana PARIS kit had the highest yield for miR-15b and -16. For miR-24 and cel-miR-39 the miRNeasy Mini Kit had the highest yield were mirVana PARIS kit was second. The lowest variability was obtained with High Pure miRNA Isolation Kit[46]. The conclusion that the yield of extracted miRNA differ by using different extraction kits or protocols are supported by other studies[47,48]. Note that Ach et al. have used paraffin-embedded tissues. Ach et al. measured 62 expression profiles isolated by three different extraction protocols: TRIzol (phenol:choloform), miRNeasy total RNA and *mir*Vana miRNA Isolation kit. There findings showed that a group of 10 miRNAs differ with more than a 2-

fold difference between extraction methods[48]. Although this difference was reported from tissue-isolated miRNAs, a 2-fold difference could lead to two different diagnosis's depending on the isolation kit. A low RNA yield could result in failure of detection of miRNA profiles. Therefore it is important that isolation is optimized to gain the maximized miRNA yield.

Isolating extracellular vesicles can be achieved with differential centrifugation. The principle of this technique is using multiple centrifugation steps. A factor which influences the number of retrieved EV's with ultracentrifuge is the viscosity. Reports show an increased viscosity will reduce the number of retrieved EV's. Suggestion was made that more viscous fluids need longer centrifuge time for compensation[49]. Lamparski et al. reported that only 5% to 25% of the starting EV's could be recovered with ultracentrifugation[50]. Alternatively, surface proteins on the EV membrane can be targeted with antibodies to isolated them. Antibodies coated to beads can hold then EV's while other components will be lost during wash steps. Disadvantage of this method is that a lower yield will be extracted[51]. Commercial kits have been developed as well. Although commercial kits allow easy isolation procedures, results need to be take cautiously because the kits are not always able to distinguish the size of the EV's[29].

<u>miRNA detection methods</u>

The most common used techniques for detection of miRNAs are qRT-PCR, hybridization based methods like microarrays and next generation sequencing. Chen et al. was the first who developed a quantitative real time PCR technique for miRNA analysis. MiRNAs are approximately 22 nucleotides long and primers used for normal PCR's are about the same length. To solve this problem Chen et al. used a stem-loop primer specific to the 3' end of the miRNA[52]. Another method for qRT-PCR is adding a poly A tail to the 3'end of the miRNA[53]. Both methods can be used in combination with pre-plated primer, enabling detection of hundreds of miRNAs at the same time (Fig.2). The qRT-PCR technique will not be able to identify new miRNAs, advantages are the specificity and sensitivity of the technique and the absolute quantification[54].

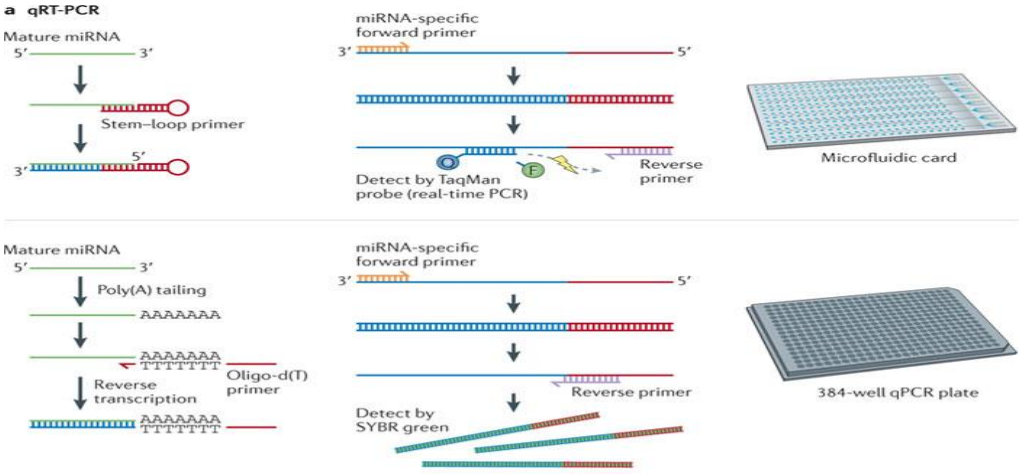

Figure 2: Quantitative reverse transcription PCr (qRT-PCR)[54]. The Taqman qRT-PCR uses a stem-loop primer specific to the 3' end of the miRNA. Amplicons generated with DNA polymerase will hydrolyse the Taqman probe. SYBR-green qRT-PCR uses a polyA-tail for reverse transcription. Generated ds-DNA binds with SYBR-green for quantification. Both techniques can be used with pre-plated primer plates for larger scale miRNA profiling.

Microar
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are based on the hybridization between a probe and the miRNA (Fig. 3). Disadvantage is the hybridization temperature. The hybridization can only be carried out at one temperature. This can result in distortion in the fluorescent signals. Microarrays have a lower specificity then qRT-PCR and will also not be able to detect novel miRNAs, but the advantage is the large numbers of miRNAs which can be profiled[54].

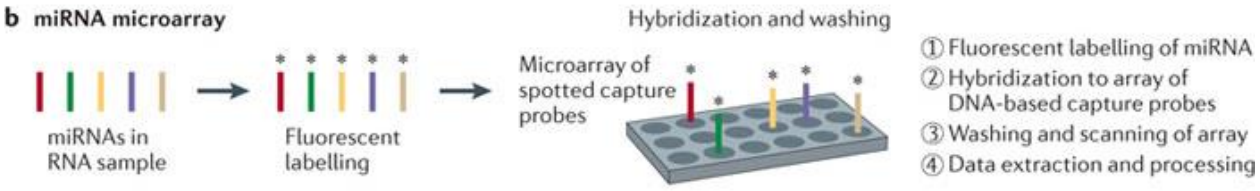

Figure 3: Hybridization based method: miRNA microarray[54]. miRNAs are tagged with fluorescent labels. Target probes are used to capture the specific fluorescent labeled miRNAs. Scanning the fluorescent spots can determine the quantification.

Next generation sequencing is the third major detection method for miRNA profiling. Next generation sequencing is using cDNA instead of miRNA (Fig. 4). To get cDNA of all miRNAs an adaptor ligation is required. Oligonucleotide adaptors are ligated to the 5' and 3' ends of the miRNA. The adaptor sequence can be used for primers to reverse transcriptase the miRNA in cDNA and for amplification of the cDNA. The adaptor allows also the cDNA to affix on a solid phase[57]. The advantages of next generation sequencing is, it will be able to distinguish very similar miRNA sequences and it can detect novel miRNAs[54].

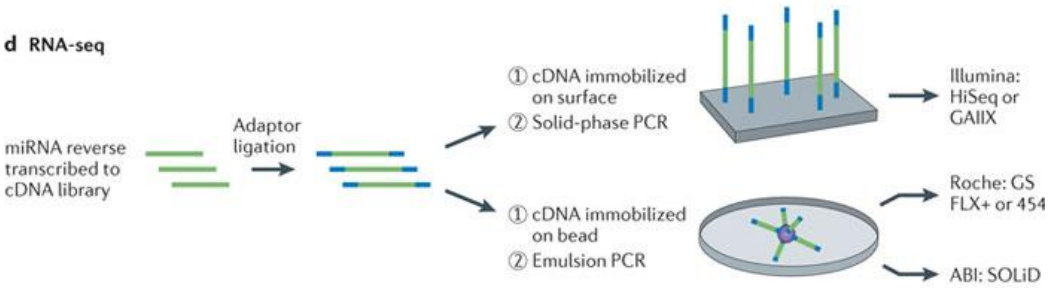

Figure 4: Next generation sequencing[54]. Reverse transcription is used to create a cDNA library of all miRNAs. The adaptors allows the cDNA to affix on a solid phase. By identification of every nucleotide separately miRNAs their sequence can be determined.

miRNAs and lung cancer

Lung cancer is the leading cause of cancer mortalities worldwide. More than 85% of all the lung cancer cases is diagnosed as non-small cell lung cancer (NSCLC). Prognosis for the 5-year survival rate is <15%. Late diagnosis is an important factor for the poor survival rate[62,63]. Circulating miRNAs could be a new method for earlier diagnosis. The last decade research showed that circulating miRNAs could give information about subclassification, prognosis and therapy strategy.

One of the first miRNAs associated with lung cancer was the *let-7* family[66]. Calin et al. reported that several *let-7* genes were located on loci which were deleted in multiple human cancers[65]. These findings were confirmed by later published results which showed that let-7 had an reduced expression in NSCLC tissues. Chin et al. concluded also that SNP's in the *let-7* gene can cause an increased risk for NSCLC[64]. Downregulation of let-7 results in the upregulation of RAS which is a known proto-oncogene[67]. Nowadays many other miRNAs have been associated with NSCLC, circulating miRNAs miR-148a, -148b, -152 and -21 have all been found in serum with altered levels[68]. Note that alterations in the miR-21 level is studied in various cancers and may not be specific for lung cancer alone. Decreased levels of miR-152 is reported earlier in lung cancer[69] as well as miR-148a[70] and miR-148b[71].

Shen et al. compared the level of miRNAs in lung cancer patients of paired tissue samples and plasma samples. First 12 aberrant expressed miRNAs were identified in NSCLC tissue compared with healthy tissue. From the 12 aberrant expressed miRNAs four were also measured with aberrant level in plasma, miRNA-21, -126, -210, and 486-5p. With these four circulating miRNAs was Shen et al. able to distinguish 58 NSCLC patients from 29 healthy controls with a yield of 86 % sensitivity and 96 % specificity. The same four miRNAs were used for identifying stage I NSCLC patients and 73% sensitivity and 96% specificity was achieved[74]. Identifying stage I NSCLC patients is important in an early diagnosis. Interestingly Shen et al. showed that miR-205 was overexpressed in tissue but did not display changes in plasma levels[74]. The overexpression of miR-205 was associated with subclassification between squamous cell carcinoma (SSC) and adenomacarcinoma (AD). SCC and AD are the two most common subtypes of non-small cell lung cancer. Subclassification between AD and SCC is important because different stages or subclasses may require different approach in therapy. Labanony et al. reported a sensitivity of 96% and specificity of 90% for distinguishing AD and SCC with miR-205 in tissue samples[72]. This result was later confirmed with similar accuracy[73]. The results of Shen et al. points out that miRNAs with aberrant expressions in tissue samples cannot just be used as circulating miRNAs. This means for miR-205 that it cannot be used as circulating biomarker.

Zheng et al. identified three other circulating miRNAs as possible biomarker in plasma. The levels of miR-155, -197 and -182 were all elevated. To evaluate the diagnostic value of these three miRNAs, 142 plasma samples were profiled. The cohort consists out of 74 lung cancer samples and 68 healthy samples. The three miRNAs were able to discriminate lung cancer from healthy samples with 81.33% sensitivity and 86.76% specificity[75]. The sensitivity and specificity are lower than previous reported data from Shen et al.[74]. But in addition Zheng et al. showed that patients with metastasis had significantly higher levels of all the three miRNAs[75].

miRNAs in cancer prognosis

The possibility to detect patients with a metastasis may have impact on the prognosis. Therefore it is interesting to see if circulating miRNAs could give information about a possible stage, size or maybe even an prediction on the survival rate. For example Yang et al. reported miR-21 in serum as potential circulating biomarker for classification of NSCLC. But besides the classification Yang et al. reported also that higher levels of miR-21 in serum was correlated with a larger size and advanced stage of the lung tumor[68]. This would suggest that miR-21 has a more prognostic value than a diagnostic value for lung cancer patients. Yanaihara et al. investigated the correlation between miR-155, let-7 and the survival rate. The data showed that patients for an adenocarcinoma that higher levels of miR-155 and decreased levels of let-7 had a poorer survival rate[76].

Hu et al. compared in two groups of patients their serum samples. The first group consists out of 30 patients who had a survival time shorter than 25 months (average was 9.54 months). The second group were 30 patients who survived longer than 30 months (average was 49.54 months). Next generation sequencing was able to detect 109 miRNAs in the longer-survival group where 101 miRNAs were found in the shorter-survival group. Comparing the groups resulted in 11 miRNAs with more than five-fold difference. In addition, let-7a and let-7g were also included because they showed a two-fold difference and the let-7 family was earlier reported to be correlated with lung cancer survival[76]. Using qRT-PCR to quantify the 13 miRNAs resulted in only four miRNAs being significantly different between the groups, miR-1, -30d, -486, and -499. Data showed that miR-489 and miR-30d had increased levels in serum and miR-1 and miR-499 decreased levels. Further research in the four circulating miRNAs showed that patients with alterations in two or more miRNA levels had an increased probability for shortened survival[85].

Correlation between miRNAs and the survival rate can have a great value for the prognosis. Kaduthanam et al. concluded in their report that high risk recurrence in early-stage lung adenocarcinoma patients was associated with increased levels of miR-142-3p in sera[86]. Zheng et al. showed that increased levels of miR-155, -197 and -182 in plasma was associated with metastasis[75]. The possibility to detect lung cancer with a metastasis has a prognostic value. And besides prognostic value the detection of metastasis may adjust the therapy strategy also. On the other hand the identification of a metastatic cancer with an unknown primary origin is often diagnosed with a poor prognosis as well. The ability to identify the primary origin from a metastatic cancer can contribute to a better prognosis. Tumors from different origin are treated with different therapies and thus the identification of the primary origin is of major importance. Therefore studies have used a set of multiple miRNA classifiers for tracing the origin of cancers of unknown primary origin. Results showed a sensitivity above 85% for an accurate diagnosis[77,78]. The prognosis and survival rate predictions based on miRNA profiles may be important in a more general sense, but the prediction if a cancer responds to a therapy may have a greater clinical value.

miRNAs in cancer therapy

A main problem in therapy failure is drug resistance. Due to drug resistance not all patients with the same tumor benefit from the same therapy. Prediction of drug resistance based on miRNA profiles could lead to a better therapy strategy. Circulating miRNAs may become useful in the prediction of drug resistance. Reports showed that aberrant levels of circulating miRNAs are associated with different drug resistances.

Apo2L/tumour necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) is a drug which can induce apoptosis in cancer cells. Not all cancers can be treated with this drugs because some are resistant. Garofalo et al. performed miRNA expression profiling analysis on NSCLC resistant versus NSCLC non-resistant cells and showed that NSCLC resistant cells have an overexpression of miR-221 and -222. Both miRNAs inhibited p27kip1 protein expression which is needed for the apoptosis[81]. This suggest that miR-211 and -222 levels could be a potential biomarker for selection of therapy.

Wei et al. reported that miR-21 plasma levels were decreased in patients who had partial remission after platinum based chemotherapy compared to patients with a stable disease or progressive disease. Therefore it was suggested that miR-21 levels in plasma correlates with sensitivity to platinum based chemotherapy[88]. The same group published later a report that confirmed this suggestion. Further investigation in miR-21 showed that miR-21 knockout in the A549/CDDP cells suppressed the drug resistance. Ectopic expression of miR-21 in the A549 cells caused a decreased sensitivity to platinum based chemotherapy. The A549/CDDP cell line is a model for platinum-based chemotherapy resistant of lung cancer. And the A549 is the parental cell line which is not resistant. To confirm the correlation between miR-21 levels in plasma and platinum based chemotherapy resistance, 58 patients who received platinum based chemotherapy were analyzed. The results showed that patients resistant to the chemotherapy had significantly higher miR-21 levels in their plasma compared with patients sensitive to the chemotherapy[87]. The results together suggests that miR-21 could have a great predictive value as circulating biomarker for drug resistance against platinum based chemotherapy.

Epidermal growth factor receptor (EGFR) activation leads to cell proliferation. Mutations in the *EGFR* gene are associated with lung cancer. Shen et al. compared miRNA plasma levels from NSCLC patients with an EGFR mutation and without a mutation. Their results showed that miR-21 and miR-10b were both elevated in patients with an EGFR mutation. In addition, their results showed also that patients with increase miR-21 levels had a better response to gefitinib, an EGFR inhibitor, but had a shorter overall survival[84].

Discussion

The discovery of miRNAs in general and in particularly circulating miRNAs could have a great impact for the diagnosis. Better therapeutic strategies based on the circulating miRNA levels could lead to improvement in therapy and maybe a more accurate prognosis can be given. The potential looks great with the possibility to detect different tumors based on expression profiles[14,15]. But also the possibility to detect the origin of metastatic cancers with a sensitivity above 85%[77,78]. The sensitivity and specificity will only increase as there will be more miRNAs associated with specific tumors. Most golden standards are depending on invasive techniques however circulating miRNAs can be obtained non-invasive from body fluids. The results show a promising future for miRNAs, but before miRNA profiling can be used as diagnostic tool there are problems which need to be considered.

Comparison between isolation methods showed a 2 fold difference for a group of 10 miRNAs between isolation kits[48]. Thus, isolation methods need to be standardized the same goes for the effect of hemolysis or co-purification of PCR inhibitors. Another major problem is the normalization of the data. Most studies compare disease against healthy samples and all the data will be filtered on the difference that is a consequence from the disease. But variations in expression profiles could be caused through a technical source as well, for example the hemolysis. For miRNAs there is not an universal control/housekeeping miRNA. Often other small RNA's are used for normalization, for example: 5S rRNA, U6 snRNA or RNU44. Chen et al. investigated the combination of let-7d, let-7g and let-7i as normalizer. Their data suggested that the combination of the three let-7 miRNAs was superior to U6, RNU44, RNU48 and miR-16[89]. But the lack of consensus has resulted in multiple normalization strategies. The advantages and disadvantages of the different normalization strategies are not clear. This could possibly affect the detection of miRNAs and thus the diagnosis.

Different specimens can lead in different expression profiles. For example miR-205 was found at higher levels in NSCLC tissue samples and reports suggested that miR-205 could distinguish adenocarcinoma for squamous cell carcinoma[72,73]. But when Shen et al. compared NSCLC tissue samples with paired plasma samples they were not able to find miR-205 in plasma at all[74]. Thus, miRNAs associated with diseases proven in different specimen cannot just be used as biomarker for circulating miRNAs until it is proven.

The identification of tumors with circulating miRNAs shows potential but in practice the idea for using this method for screening would be expensive and give a lot of work. Subclassification based on circulating miRNAs could be more useful. For example the subclassification of NSCLC tumors can be done with immunohistochemistry (IHC) but this requires invasive techniques to obtain tissue samples. Advantage for using circulating miRNAs is that it can be obtained with minimal invasive techniques. The distinction between AD and SCC was associated with altered miR-205 levels but only it was possible to detect this in tissue samples and not with circulating miRNA[74]. Thus, to classify NSCLC into AD or SCC with a circulating miRNA further research is needed. Different tumors must be treated with a different therapies so subclassification is important for the therapy.

Alterations in circulating miRNA levels could have prognostic value but can give also information for therapy strategy. Higher serum levels of miR-21 have been associated with a larger size and advanced stage[68]. Corresponding to these findings higher levels of miR-21 in plasma reduced the overall survival for NSCLC patients[84]. The same study concluded also that patients with a lower level of miR-21 were less sensitive to gefitinib. But on the other hand patients with decreased levels of miR-21 in plasma were more sensitive to platinum based chemotherapy[88]. If the sensitivity to gefitinib and platinum based chemotherapy are linked to each other is unknown. But it is clear that miR-21 plays an important role in the tumorigenicity and that it can have a prognostic and therapeutic application. However miR-21 is one of the most common studied miRNAs in various cancers and therefore not tumor specific. Hu et al. was able to find four specific miRNAs, miR-1, -30d, -486, and -499, for the prognosis of NSCLC patients. Hu et al. concluded that patients with two or more aberrant levels in serum had an increased probability for shortened survival[85].

More tumor specific miRNAs can only contribute to a better diagnosis, prognosis and therapy strategy. With only miR-21 as circulating miRNA not a single tumor can be distinguished. For every tumor or subclass a specific circulating miRNA profile need to be found. For the prognosis and therapy there is in most cases already a specific tumor known and then miR-21 can be used. But also for the prognosis and therapy strategy, if more specific miRNAs markers are known a better prognosis and therapy can be given. Or even better an combination of circulating miRNAs rather than a single miRNA. A last major problem is the reliability of miRNAs associated with specific tumors. Most miRNAs are only once or twice associated with a specific tumor. And therefore more research is needed to see if a specific miRNAs is really specific for a tumor. If other laboratories are able to obtain the same results, that would mean that specific miRNA profile can be associated with specific tumor and that it is reproducible.

In conclusion the potential of circulating miRNAs as biomarker is promising. The information miRNAs can give about diseases as classification, prognosis and therapy strategies is hopeful. However from technical point of view there are still limitations need to be considered. Further studies need to investigate the best standardization and normalization method to establish a reliable and reproducible diagnostic tool. To establish this goal more research is needed to but it seems inevitable that circulating miRNAs will be used in the future for diagnostic purpose.

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