International Journal of Pharmaceutical and Medical Research
Volume – 3 Issue – 2 April 2015
Website: www.woarjournals.org/IJPMR *ISSN: 2348-0262*

Advance in development of serum micro RNAs as novel biomarker for tumor

Birendra Kumar

Department of Biochemistry, Bundelkhand University, Jhansi-284128, India
Therapeutic Monoclonal Antibody Laboratory, National Institute of Biologicals, Noida-201309, India

Corresponding author address:
Therapeutic Monoclonal Antibody Laboratory, National Institute of Biologicals, Noida-201309, India

Abstract: ***Serum and other body fluids of human are wealthy sources for the detection of novel biomarkers, which can be considered in routine medical diagnosis. MicroRNAs are a class of small non-coding RNAs that regulate gene expression at the posttranscriptional level by either degrading or blocking translation of messenger RNA targets. The deregulation of microRNAs has been associated to tumor progression and cancer development. Lately, the detection of microRNAs in serum and other body fluids of human include adequatelely constant microRNA autographs. Recent expression profiling studies have discovered that microRNAs participate significant regulatory roles in a multiplicity of cellular functions as well as intending at the detection on novel non-invasive biomarkers. In this article, I review the current literature on serum microRNAs in different tumor types and the approaches used to detect and quantify these molecules. Moreover, I précis the knowledge about the mechanism of microRNAs discharge and the putative functional roles of circulating microRNAs. There remain many challenges in this domain, circulating microRNAs have the prospective to be constructive for the diagnosis and prognosis of tumor ailments.***

Keywords: Biomarker, MicroRNA, Serum, Tumor.

1. Introduction

Cancer is the most dreaded disease haunting the mankind since time immemorial. It affects everyone – the young and old, the rich and poor, men, women and children – and represents a tremendous burden on patients, families and societies [1]. Cancer is the third leading cause of mortality after cardiovascular and infectious diseases in the world. Although we have made great advances in the understanding of cancer biology and pathogenesis as well as in the development of new targeted therapies, the progress in developing improved early diagnosis and screening tests has been inadequate. As a result, most cancers are diagnosed in advanced stages, which lead to poor outcomes. Now, intense research is focused on seeking specific molecular changes that are able to identify patients with early cancer or precursor lesions. Biological samples such as blood, serum, stool, pancreatic juice or urine, as well as both DNA and RNA, have been analyzed for tumor-specific changes. However, due to the simplicity of getting a blood sample, easily testable biomarkers found in blood serum would be especially useful. Furthermore, the unique patterns of disordered miRNA expression in each type of cancer, their stability in serum [2], [3] and their role as biomarkers of disease risk due to inherited polymorphisms suggest that miRNAs may potentially serve as novel molecular biomarkers for clinical cancer diagnosis.

Ambros and colleagues (1993), discovered a gene, lin-4, which affected development in Caenorhabditis elegans (C. elegans); they found that its product was a small non-protein coding RNA, microRNA (miRNA) [4]. miRNAs are endogenous non-protein-coding short RNAs of 21-23 nucleotides [5], [6]. It was initially discovered in *Caenorhabditis elegans* and thousands have been identified in many organisms, including human, mammals, invertebrates, insects, plants and viruses. In humans, miRNAs play important roles in cellular physiology, development, and disease by negatively regulating gene expression [5], [6]. miRNA biogenesis requires several post-transcriptional processing steps to yield the functional mature miRNA [7]. Currently, there are 940 mature human miRNA sequences listed in the miRNA registry (Sanger miRBase release 15; http://www.mirbase.org/).

Over the past several years, many miRNAs have been investigated in various human cancers [8]. The deregulation of the expression of miRNAs has been shown to contribute to cancer development through various kinds of mechanisms, including deletions, amplifications, or mutations involving miRNA loci, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inhibition of processing. miRNA expression profiling is of increasing importance as a useful diagnostic and prognostic tool, and many studies have indicated that miRNAs act as either an oncogene or a tumor suppressor. Recently, the discovery of miRNAs as novel biomarkers in serum or plasma represented a new approach for diagnostic screening in blood. Since current approaches to cancer screening are invasive and it is difficult to detect cancer in its early stages, it is important to understand the characteristics of secretory miRNAs and their usefulness in cancer detection. In this article, I review and assess the potential efficacy of serum miRNAs in cancer therapeutics and diagnosis.

2. Cell-free microRNAs and feasible discharge mechanisms

Although miRNA presence is pertinent for the regulation of cancer-associated genes in tissues, the possibility to extract and

reliably determine cell-free miRNA content in body fluids like serum was first shown in 2008 [9]. This finding was confirmed by a subsequent study revealing that miRNAs are enriched in the small RNA fraction isolated from serum samples [2]. Serum specimens contain high volumes of protein, and therefore extraction requires a modified protocol that adds more denaturing solution such as Qiazol or Trizol [10]. Additionally, considering the small amount of RNAs from serum or plasma samples, a routine spike-in synthetic non-human *C. elegan* miRNA (e.g. Cel-miR-39) was used after the initial serum denaturation step to serve as the internal quality control [11]. Considering that profiling of circulating miRNAs are frequently confounded by cellular miRNAs, I believe that cell-free circulating miRNAs from exosomes will be a better starting material for profiling studies. Unfortunately, cross-contamination between cells and exosomes seem to be unavoidable. The alternative might be to enrich exosome fractions and eliminate cell fractions. In order to enhance the specificity of potential circulating miRNAs, I recommend the blood plasma and serum preparation protocol modified by Duttagupat et al. [12]. For better targeting the tumor-derived exosomes, tumor-antibody coupled magnetic beads could be used to enrich the tumor relevant miRNAs [13]. Once potential exosomal miRNA biomarkers are identified, they could be validated by using whole blood or unfractioned serum or plasma. Cell-free miRNAs in body fluids are stable under harsh conditions including boiling, low/high pH, extended storage and multiple freeze-thaw cycles [2], [3], [14]-[16]. In contrast, synthetic miRNAs were found to be quickly degraded by the high levels of RNAse activity in plasma [3]. Filtering and differential centrifugation experiments suggest that miRNAs are not derived from cells circulating in the blood [3].

At present, there are at least two possible explanations for the stability and origin of circulating miRNAs: One hypothesis is that passive release occurs during tissue injury. For example, miRNA-208 was shown to be exclusively expressed in the heart and was measured in the serum after heart tissue injury [17]. The same unspecific release could also exist in cancer, since the high rate of proliferation and cell lysis in tumors might contribute to the abundance of miRNAs in the blood stream. Alternatively, miRNAs are contained in small particles and are therefore protected against RNase activity. Recently, it has been shown that a transfer of mRNA and miRNA between cells can be accomplished through microvesicles [18]. These are small (50 nm to 100 nm) particles, which are shed from the cell plasma membrane into the extracellular space and released into the blood stream [19], [20]. Microvesicles are derived from different cell types, e.g. reticulocytes, dendritic cells, B/T cells and mast cells [21]-[25]. Additionally, it was shown that non-hematopoietic cells like intestinal epithelial cells and neuroglial cells are capable to release microvesicles [26], [27].

3. Circulating miRNAs function in tumor

Current research has revealed evidence showing that miRNAs play roles in the initiation and development of tumor [28]. Some of these miRNAs modulate expression of known oncogenes or tumor suppressor genes, whereas others function as so-called onco-miRs or tumor-suppressor-miRs. Circulating microvesicles have been recognized for many years, but their generation and physiological roles are still incompletely understood. Microvesicles, also known as exosomes, are supposed to be important for cell-cell communication. However, the role of incorporated miRNA molecules is unclear. Hunter et al. compared the expression levels of miRNAs from microvesicles with that of platelets and peripheral blood mononuclear cells in healthy individuals [29. Significant differences were found and target prediction demonstrated that the majority of the miRNAs from the microvesicles are involved in the regulation of hematopoiesis and cellular differentiation [29]. Recently, the mechanism of microvesicle based miRNA release was reported to involve ceramide-dependent secretory machinery [30]: Kosaka and colleagues showed that miRNA abundance changes after overexpression and inhibition of a rate-limiting enzyme involved in ceramide biosynthesis [30]. Entertainingly, they also found that miRNAs are transported in microvesicles and exert gene silencing in recipient cells. The recent data suggest that cells can actively secrete endogenous miRNAs. However, it remains unclear whether there is a specific mechanism leading to an increase of certain selected miRNAs. Although the exact mechanism for microvesicle formation and nucleic acid incorporation is still unknown, exosomes seem to have important roles in cell-cell communication [20]. Therefore, the contained miRNAs could also have important functions in tumor development and progression: In 1979, it was shown that tumor related exosomes are present in the blood of women suffering from ovarian cancer [31]. Recently, the quantity of tumor-derived exosomes in the peripheral circulation has been found to be highly correlated with ovarian cancer stages [14]. Moreover, the miRNA content of tumor cell-derived exosomes is correlated to the miRNA level in the primary tumor [14], [32]. Skog et al. reported that glioblastoma-derived RNA contained in microvesicles is functional and is taken up by and processed in human brain microvascular endothelial cells (HBMVEC) in cultures [33]. This lead to the hypothesis that tumor cells use exosomes to transport genetic information, including miRNAs, to surrounding cells and thereby support tumor growth and progression [33]. If this hypothesis holds true, miRNAs could be suitable candidates to manipulate the microvesicle's target cells by regulating their RNA stability and translation. Moreover, circulating miRNAs might modulate immune responses [34], [35]: For example, microvesicles derived from human melanoma and colon cancer can promote tumor growth and immune escape by mediating the differentiation of monocytes towards TGFb-secreting myeloid suppressive cells [34]. However, it has not been investigated if these effects are mediated by the miRNAs contained in the microvesicles.

4. Circulating miRNAs reflect pathological or physiological alterations

Circulating miRNAs can be readily detected in serum [17], plasma or whole blood [36]. Using Solexa sequencing technology, Chen et al. detected a great amount of small RNAs, 21-23nt length in both serum and whole blood samples from normal individuals, as well as patients with colorectal, non-small cell lung cancer and diabetes [2]. Another study confirmed the presence of small RNAs (18-24 nt) in plasma from the total RNA extracted from human plasma using radioactive labeling method [3]. Followed by cloning and sequencing of those small RNAs, over 93% of the sequences matched known miRNAs, and thus further confirmed that the majority of small RNAs isolated from plasma were indeed miRNAs. In addition, some randomly selected miRNAs were found to be expressed consistently in both serum and plasma samples from humans, as well as in other species, such as rats,

mice, claves, bovine fetuses and horses [2]. MiRNAs are also notably stable in serum and plasma samples [2], [3], [17], [36], [37]. Chen et al. found that isolated serum miRNAs can survive the treatment of RNase A, compared to other endogenous RNAs such as 18s rRNA, 28s rRNA, GAPDH, β-actin and U6 [2]. Most serum miRNAs maintain considerable expression levels after 3 hours or overnight RNase A treatment; however large RNAs were degraded following 3 hours of RNase A treatment. Furthermore, repeat freeze-thawing cycles [2] and low/ high pH solution [2] treatments did not affect serum miRNAs. Similarly, plasma miRNAs could remain stable in room temperature for 24 hours and eight freeze-thaw cycles; however, synthetic miRNAs were rapidly degraded in plasma [3]. This indicates that the endogenous plasma miRNAs in RNase-enriched circulating system exist in a form that is resistant to plasma RNase activity. In healthy individuals, the levels of cell-free miRNAs present in sera are stable [2], [15]. Under healthy conditions, the serum miRNA profile is similar to that of circulating blood cells [2]. Thus, alterations of serum miRNA levels may be indicative of physiological or pathological changes and may possibly be used as surrogate biomarkers [38]. For example, circulating miRNAs were found in the sera of pregnant women [15]: miRNA-526a, miRNA-527 and miRNA-520d-5p showed a considerably high fold-change and could be used to distinguish pregnant from non-pregnant women with high accuracy. Moreover, placenta derived miRNAs (e.g. miRNA 141, miRNA 149, miRNA 299 5p and miRNA 517a) are detectable in the maternal plasma, and their concentrations decrease directly after childbirth [39], [40]. Therefore, miRNAs have been discussed as novel noninvasive markers for prenatal diagnosis [39]. As to pathological changes, tissue specific miRNAs were analyzed in the blood stream as markers for myocardial injury and drug induced liver injury: A rat model of acute myocardial infarction demonstrated that the plasma levels of the cardiac-specific miRNA-208 and miRNA-499 are increased in this disease [41]. These miRNAs are also elevated in the plasma of human patients with acute myocardial infarction [41], [42]. Drug induced liver injury, a frequent side effect which significantly influences the patient's health and treatment costs is associated with increased plasma levels of miRNA 122 and miRNA 192 in a mouse model [43]. Cell-free miRNAs are also associated with inflammatory diseases [44], [45]: Circulating miRNA-146a and miRNA-223 were significantly reduced in septic patients when compared to patients with a systemic inflammatory response syndrome or healthy controls [44]. Furthermore, reduced plasma levels of miRNA 132 were observed in patients with rheumatoid arthritis and osteoarthrosis compared to healthy controls [46].

5. Serum miRNAs as prospective biomarkers for tumor diagnosis

It is reported that miRNAs could be an ideal class of blood-based biomarkers for tumor detection because: (i) miRNA expression is frequently dysregulated in tumor [47], [48], (ii) expression patterns of miRNAs in human cancer appear to be tissue-specific [49] and (iii) miRNAs have unusually high stability in formalin-fixed tissues [50]. This third point led us to speculate that miRNAs may have exceptional stability in plasma and be promising biomarkers for diagnosing human cancers. The availability of novel biomarkers could improve diagnosis and the clinical management of cancer. A perfect biomarker should be easily accessible in a noninvasive manner. Therefore, miRNA profiles in serum and plasma samples from cancer patients have been screened to identify novel biomarkers for the diagnosis of tumors (summarized in Table 1).

5.1 Serum miRNAs in lymphoma, acute leukemia and glioblastoma

A malignant disorder of lymphoid progenitor cells, affects both children and adults, with peak prevalence between the ages of 2 and 5 years. The diagnostic and prognostic potential of miRNAs in the serum of patients with diffuse large B-cell lymphoma (DLBCL) was shown by studies of Lawrie et al. [51]. They determined the expression levels of three tumor-associated miRNAs in serum samples from DLBCL patients and healthy controls and found that the miRNAs miR-155, -210 and -21 were upregulated in patient sera (see also Table 1 for an overview). Moreover, DLBCL patients with high miR-21 serum levels showed increased relapse free survival but not overall survival. miRNAs do not have to be specific for a certain cancer type. Skog et al., for example, showed that miR-21 is also elevated in the serum of patients with glioblastoma tumors, consistent with the fact that this miRNA is overexpressed in glioblastoma tumor cells [52]. The results of these studies are promising, but sera from more DLBCL and glioblastoma patients must be tested before these findings can be generalized. Additionally, it is yet unclear how tumor miRNAs find their way into the bloodstream. The detected extracellular miRNAs might be derived from dying tumor cells, from tumor cells that have been lysed, from cells infiltrating the lymphomas, from other tissues affected by ongoing diseases, or because the tumor cells actively secrete miRNAs into the surrounding environment. Further studies with larger sample numbers and ideally, monitoring and follow-up of patients within clinical trials should allow detection of miRNA expression before, during and after therapy and could lead to

Table 1. Serum miRNAs as a biomarker for different human tumors

Type of tumor	miRNAs	Contributors
Diffuse large B-cell lymphoma	miR-21, miR-155, miR-210	[56]
Glioblastoma	miR-21	[57]
Acute myeloid leukemia Acute lymphoblastic leukemia	miR-92a	[58]
Oral and squamous cell cancer	miR-184 miR-31 miR-24	[62] [63] [64]
Breast cancer	miR-155 miR-195	[68] [69]
Lung cancer	miRNAs 63 absent in healthy controls 28 miRNAs absent in lung cancer patients miR-25, miR-223 miR-17-3p, miR-21, miR-106a, miR-	[75] [70]

	146, miR-155, miR-191, miR-192, miR-203, miR-205, miR-210, miR-212, miR-214 miR-1,miR-30d, miR-486, miR-499	[71]
Colorectal cancer	miR-17-3p,miR-92 miR-29a, miR-92a 69 miRNAs absent in healthy controls	[73] [74] [75]
Gastric cancer	miR-17-5p, miR-21 miR-106a, miR-106b, let-7a	[76]
Hepatocellular carcinoma	miR-500	[81]
Pancreatic cancer	miR-21, miR-155, miR-196a, miR-210 miR-210	[82] [83]
Ovarian cancer	miR-21, miR-29a, miR-92, miR-93, miR-99b, miR126, miR-127, miR-155 miR-141	[86] [87]
Prostate cancer	miR-16, miR-34b, miR92a, miR-92b, miR-103, miR107, miR-197, miR-328, miR-485-3p, miR-486-5p, miR-574-3p, miR-636,miR640, miR-766, miR-885-5p	[91]

the identification of useful biomarkers in cancer. In a related study, Tanaka et al. investigated whether the expression levels of specific miRNAs differ between leukemia patients [several types of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)] and healthy individuals [53]. The miRNA expression profile in human plasma was determined using microarray analysis, and the researchers found high expression of miR-92a in tumor cells of acute leukemia. One miRNA, miR-638, was the most highly expressed miRNA in healthy individuals regardless of sex and age, suggesting that miR-638 is physiologically necessary in blood and might be a useful internal control for quantification of miRNAs in plasma. The authors also showed that the ratio of miR-92a/miR-638 in plasma could be a very sensitive biomarker for AML and ALL.

5.2 Serum miRNAs in oral and squamous cell cancer

Oral cancer consistently ranks as one of the ten most frequently diagnosed cancers in the world [54] with over 363,000 cases reported annually worldwide, and mortality rate of about 50% [3], [55], [56]. Studies by Wong and colleagues identified by qRT-PCR analysis of laser microdissected cells from four tongue carcinomas and paired normal tissues an overexpression of miR-184 that was further validated in 20 paired tongue squamous cell carcinomas and normal [57]. In addition to showing an effect of miR-184 inhibition on cell proliferation rate and apoptosis in tongue squamous cell carcinoma cell lines, they also demonstrated that plasma miR-184 levels were significantly higher in tongue squamous cell carcinoma patients than in normal individuals when normalized to the control miRNA miR-16. Interestingly, the plasma levels of miR-184 were significantly reduced after surgical removal of the primary tumors. Importantly, plasma miR-184 could be detected in both early and advanced tongue squamous cell carcinoma patients, indicating a possible use of this miRNA for early detection of this cancer type. Oral squamous cell carcinoma (OSCC) is one of the most frequent carcinoma worldwide. Liu et al. [58] therefore investigated the role of miR-31, which was beforehand known to be involved in OSCC, in plasma and saliva of OSCC patients compared to an age and sex-matched control population. qRT-PCR analysis indicated that OSCC patients had significantly higher levels of miR-31 in their pre-operative plasma than controls. Interestingly, after resection, 88% of patients showed a varying degree of decrease in plasmamiR-31 levels. The same trend was also observed for miR-31 levels in saliva, as miR-31 was increased in OSCC patients and a similar decrease was apparent in patients after tumor resection. Regions on chromosomes 9 and 19 are frequently altered in patients with head and neck squamous cell carcinoma. As the genes encoding miR-24 are located in these regions, Lin et al. [59] analyzed miR-24 plasma levels in OSCC patients and control individuals. qRTPCR analysis indicated a significant 2.4-fold higher miR-24 plasma level in OSCC patients than in controls, yielding a predictive power and an accuracy of 0.82 and 0.73 for miR-24 plasma levels in distinguishing malignant and non-malignant states. Although the estimation of each miRNA's diagnostic power is limited by the small sample size in all three OSCC studies, the combination of miR-184, miR-31 and miR-24 as OSCC serum biomarkers could yield very attractive candidates for validation in large-scale follow-up studies.

5.3 Serum miRNAs in breast cancer

Breast carcinoma, which is the second most prevalent cancer in women, is diagnosed in >200,000 woman in the USA every year. Early detection is a major factor contributing to the 2.3% annual decline in breast cancer death rates over the past 10 years [60]. Nonetheless 40,480 women in the USA were projected to die from breast cancer in 2008 [61], in part because currently available breast cancer screening tools such as mammography and breast examination miss 10-40% of early breast cancers and are least effective in detecting cancer in young women, whose tumors are often more aggressive. One of the most frequent cancers in women is breast cancer. While mammography is currently the gold standard diagnostic tool, it is not without limitations, including use of ionizing radiation and a considerable false positive rate [62]. For routine evaluation of breast tumors, estrogen receptor (ER) (for predicting response to endocrine therapies) and HER2/neu are currently used. In a pilot study for potential miRNA serum

biomarker, Zhu et al. compared the expression levels of three miRNAs in serum samples of breast cancer patients and healthy subjects [63]. While the expression of all three miRNAs was similar in samples from healthy women compared to those with breast cancer, women with progesterone receptor-positive tumors had highermiR-155 expression than women with tumors that were negative for this receptor. This indicates that in addition to the differentiation of normal from diseased states, serum miRNA signatures could be used to determine into which prognostic groups subjects should be placed and how they might respond to therapy. Heneghan et al. [64] studied 7 candidate miRNAs in tissue and blood specimens of 148 patients with breast cancer and 44 age matched and disease free control individuals. They could show that increased miR-195 levels in breast tumors were reflected in serum of breast cancer patients and that this level was significantly higher than in control individuals. Interestingly, they could also show that miR- 195 and let-7a expression in the blood decreased significantly postoperatively to levels comparable with control subjects, indicating a potential use of these miRNAs to assess successful curative tumor resection. Analyzing circulating miRNAs also allowed the authors to correlate clinic pathological variables: lymph node positive patients were found to have significantly lower levels of circulating let-7a compared with those with lymph node negative disease, while higher circulating levels of miR-10b and miR-21 were observed in patients with ER negative disease, compared with those with ER positive breast cancer.

5.4 Serum miRNAs in lung, colorectal and gastric

Lung cancer is the leading cause of cancer deaths worldwide, and approximately 80% of the patients have non-small-cell lung cancer (NSCLC). The potential application of miRNAs as biomarkers in lung cancer is encouraged by studies of Rabinowits et al. that suggested that circulating exosomal miRNA might be a useful screening parameter for lung adenocarcinoma [65]. The miRNA signatures of peripheral circulating exosomes parallel those of tumors, indicating that miRNA profiling can be performed in the absence of tissue and accurately reflect the miRNA profile of the tumor. This approach could potentially be extended to the screening of asymptomatic individuals and to monitoring disease recurrence. A Solexa deep sequencing approach followed by individual qRT-PCR analysis was used by Hu et al. [66] to investigate the role of serum miRNA in predicting prognosis of NSCLC by analyzing the difference in serum miRNA levels of patients with longer-compared to shorter survival. Of the 109 miRNAs that were detected by sequencing from the longer-survival group (compared to 101 miRNAs that were detected from the shorter-survival group), miRNAs were selected for additional qRT-PCR validation that were well detectable and showed an at least five-fold altered expression between the two pooled samples. This resulted in the identification of eleven serum miRNAs, and the levels of four miRNAs (miR-486, miR-30d, miR-1 and miR-499) were significantly associated with overall survival. The obtained results from the training set could be projected onto the test set, as the four-miRNA signature was also consistently an independent predictor of overall survival in this set. High serum expression levels of miR-486 and miR-30d and low serum expression levels of miR-1 and miR-499 were all individually associated with unfavorable survival. Patients already carrying two or more high-risk miRNAs had a significantly increased probability of shortened survival than those carrying zero or one high risk miRNA in both sample sets, suggesting that either four miRNAs individually or the four-miRNA signature as a group may be used as biomarkers to predict the lung cancer survival in this study population. To generalize the usefulness of these serum miRNA signatures, further studies in different ethnic populations combined with analysis of therapy response can be envisioned.

Colorectal cancer is the third commonest malignant neoplasm worldwide and the fourth leading cause of cancer-related deaths worldwide [67]. Colorectal cancer is, if detected early, a highly curable disease. As an alternative to the invasive colonoscopies and the non-invasive, but of limited sensitivity and specificity, fecal occult blood test, Ng and colleagues searched for a potential biomarker for colorectal cancer and studied a panel of 95 miRNAs by quantitative RT-PCR (qRT-PCR) analysis [68]. In an initial, small-scale study, five miRNAs were found to be upregulated in plasma and tissues samples from colorectal cancer patients when compared to healthy controls. All miRNAs were validated in a larger cohort and two miRNAs, miR-17-3p and miR-92, were also found to be significantly upregulated in an independent, large set of plasma samples from colorectal cancer patients compared to patients with gastric cancer, inflammatory bowel disease and healthy controls. miR-92 yielded a sensitivity of 89% and a specificity of 70% in discriminating colorectal cancer patients from control subjects. Interestingly, after surgical resection of tumors, the elevated plasma levels of miR-17-3p and miR-92 were markedly reduced, possibly indicating that the elevated miRNA levels were caused by the colorectal cancer cells. Elevation of miR-92 was independent of the tumor volume, metastatic status and tumor stage, suggesting that miR-92 could be useful in the early detection of colorectal cancer. Similar results were obtained in a study by Huang et al. [69] that showed that the levels of miR-29a and miR-92a in plasma samples from patients with advanced colorectal neoplasia were significantly higher than those of healthy controls. Although a different normalization control and more patients with earlier stages of colorectal neoplasias were used in this study, the results were comparable to that of Ng et al. The promising results of both studies still need to be confirmed in larger cohorts to address the questions of whether the elevation in miR-92 is specific for colorectal cancer, if it is specific for certain stages of colorectal cancer and if miR-92 can be used to differentiate the familial type of colorectal cancer from sporadic forms. A more comprehensive analysis of miRNAs in serum was performed by Chen et al. in a study that was the first to comprehensively characterize blood miRNA profiles from healthy individuals and patients with several different cancers [70]. First, they demonstrated that serum miRNAs are stable under harsh conditions like boiling, low or high pH, extended storage, or freeze–thaw cycles and that they are even resistant to RNase A digestion. They further demonstrated that in addition to humans, miRNAs are also present in the serum and plasma of animals such as mice, rats, bovine fetuses, calves, and horses. Furthermore, they reported that miRNAs also exist in other body fluids (urine, tear, ascetic fluid, and amniotic fluid) and, importantly, they showed that miRNA levels in serum are stable, reproducible, and consistent among individuals of the same species. Employing the Solexa deep sequencing technique, they first identified approximately 190 known miRNAs in the serum of healthy subjects. Most of the miRNAs were detected in both serum and blood cells, whereas only a small number of miRNAs were uniquely present in

either serum or blood cells. As a second step, they determined that patients with lung cancer, colorectal cancer and diabetes had specific serum miRNA profiles. In the sera of lung cancer patients, the miRNA expression profile was significantly different compared to healthy subjects, with 28 miRNAs missing and 63 new miRNA species detected. Interestingly, lung cancer patients also showed differences between the miRNAs found in serum and blood cells, which is in striking contrast to healthy subjects, in whom serum and blood cells essentially share the same miRNA profile. Two highly expressed miRNAs in lung cancer, miR-25 and miR-223, were analyzed by qRTPCR and confirmed for their ability to serve as blood-based biomarkers for lung cancer in an independent trial of 75 healthy donors and 152 cancer patients. Colorectal cancer patients also had a significantly different serum miRNA signature compared to healthy subjects. While some of these miRNAs were in common with those found in lung cancer sera, there were also some miRNAs specific to only lung cancer or colorectal cancers. Compared to healthy subjects, diabetes patients also had a significantly altered serum miRNA expression profile, although the change was not as drastic as that in cancer patients. Surprisingly, however, diabetes patients and lung cancer patients shared a large number of common serum miRNAs that were not found in healthy subjects.

Gastric cancer (GC) is the second leading cause of cancer-related death in the world. Patients with advanced GC often develop recurrent disease, even after extended radical resections, and, consequently, they show extremely poor survival rates. Serum tumor markers, such as carcinoembryonic antigen and carbohydrate antigen 19-9, have been used as convenient diagnostic assays in GC, although they lack sufficient sensitivity and specificity to facilitate early detection of cancer. Tsujiura et al. [71] therefore examined plasma miRNA concentrations from patients with gastric cancer to assess their clinical application for diagnosing and monitoring diseases. Initial studies comparing the miRNA expression of four miRNAs (miR-17-5p, miR-21, miR-106a and miR-106b) that have been reported to be upregulated in GC, and let-7a, which has been reported to be down regulated in GC, in plasma and primary GC tumor tissues demonstrated that both samples showed similar tendencies concerning the expression of miRNAs in almost all cases. The authors measured and compared circulating miRNAs in paired plasma samples before and one month after surgical removal of the tumor and found that plasma concentrations of miR-17-5p, miR-21, miR-106a, miR-106b were significantly higher in GC patients than controls, whereas let-7a was lower in GC patients. To improve the sensitivity and specificity of these plasma miRNAs for use as diagnostic biomarkers, miRNA expression ratios were calculated by dividing the plasma concentration of the four upregulated miRNAs by that of the down regulated miRNA, yielding a robust diagnostic tool. These miRNA ratios could also become powerful and useful for confirming the completeness of tumor removal and for evaluation of the efficacy of additional anti-cancer therapies.

5.5 Serum miRNAs in hepatocellular and pancreatic cancer

Hepatocellular carcinoma (HCC) is responsible for significant morbidity and mortality in cirrhosis and also accounts for between 85% and 90% of primary liver cancer [72]-[74]. Most of HCCs in the world occur in the setting of cirrhosis and over half-million of people develop liver cancer every year and an almost equal number die of it [72], [73], [75]. To understand the role of miRNAs in liver development, Yamamoto et al. studied murine liver differentiation and described miR-500 as an oncofetal miRNA that is highly expressed in murine fetal liver, even more than in normal adult liver [76]. Translating their findings to humans, they showed that miR-500 was abundantly expressed in several human liver cancer cell lines and aberrantly expressed in HCC tissue. Most importantly, an increased amount of miR-500 was found in the sera of HCC patients. Interestingly, elevated serum levels of miR-500 in some HCC patients returned to normal after surgical removal of the tumor.

Pancreatic cancer, the fourth most common cancer in the United States, remains one of the most lethal malignancies. As most cases are diagnosed after metastatic spread, the average 5-year survival rate is below 5%. In search of novel biomarkers, Wang et al. described the combined analysis of the levels of four plasma miRNAs that are known to be overexpressed in pancreatic cancer tissues (miR-21, miR-210, miR-155, and miR-196a). The analysis of these four miRNAs in plasma samples from 28 cancer and 19 control samples allowed, when combined, the discrimination of pancreatic adenocarcinoma patients from normal healthy individuals with a fairly good sensitivity of 64% and 89% specificity [77]. No significant differences in the plasma levels of the four miRNAs, both individually and in combination, could be observed for the cancer samples at different stages of the disease. One of the four miRNAs of the Wang et al. study, miR-210, was also investigated in plasma samples from newly diagnosed pancreatic cancer patients and age-matched non-cancer controls by Ho et al. [78]. Using an initial test set of 11 pancreatic cancer patients and 14 age-matched controls and a validation set of 11 pancreatic cancer patients and 11 controls, miR-210 was also reliably detected and quantified in this study, with a statistically significant four-fold increase in expression in pancreatic cancer patients compared with normal controls.

5.6 Serum miRNAs in ovarian cancer

In 2008, it was expected that 20,180 women will be diagnosed with ovarian cancer and 15,310 will succumb to the disease [79]. Ovarian cancer is a devastating illness in which only 20% of patients are diagnosed with stage I disease [80]. The poor prognosis associated with ovarian cancer is multi-factorial; a lack of minimally invasive, early detection tests, subtle symptom development and tumor chemo-resistance. The use of miRNA signatures of tumor-derived exosomes as a diagnostic biomarker for ovarian cancer was convincingly demonstrated by Taylor and Gercel-Taylor [81]. Tumor-derived exosomes were specifically isolated by a modified magnetic activated cell sorting (MACS) procedure that used anti-epithelial cell adhesion molecule (EpCAM) antibodies. The authors first showed that the level of circulating, tumor-derived exosomes in serum is strongly increased in women with invasive ovarian cancer compared to women with benign ovarian tumors or healthy controls. In addition, the levels of circulating, tumor-derived exosomes increased in parallel to the stage of disease. Further, they demonstrated by miRNA microarray profiling that the 218 miRNAs that were identified in tumor samples were also identified in circulating exosomes and that 31 of these miRNAs are overexpressed in the circulating exosomes as compared to the tumor samples. To define the significance of overexpressed miRNAs in tumor samples as biomarkers, a larger scale study including additional confounding factors will

need to be performed. Differences in serum miRNAs between healthy controls and ovarian cancer patients were also reported by Resnick et al. [82]. They sought for an alternative or complementary diagnostic approach in addition to trans-vaginal ultrasound and serum CA-125 levels for women at high risk for ovarian cancer. This would be of great importance because CA-125 remains a poor marker for early stage disease and has a documented sensitivity of 40%. miRNAs might therefore serve as early detection biomarkers in patients with normal CA-125 levels. Using a qRT-PCR platform, they identified 21 miRNAs that were differentially expressed between normal and patient serum. Analyzing these miRNAs in more detail, five miRNAs were found to be overexpressed and three miRNAs were decreased in the serum of ovarian cancer patients compared to controls, establishing a possible set of miRNAs as biomarkers for ovarian cancer.

5.7 Serum miRNAs in prostate cancer

Prostate cancer is of increasing significance worldwide due to the increasing aging population. In the U.S. alone, 1 in 6 men will develop prostate cancer in their lifetime; 1 in 30 men will die of this disease. According to the American Cancer Society, Prostate cancer is a major health concern for U.S. men resulting in approximately 218,000 new cases and about 32,000 deaths in 2010 [83]. In spite of the low known incidence of prostate cancer in developing countries, its incidence and mortality tends to increase continually [84]. A promising study by Mitchell and colleagues demonstrated that serum miRNA levels can be used to distinguish patients with prostate cancer from healthy controls [85]. First, they confirmed the finding that despite the presence of RNase activity in plasma, endogenous miRNAs are present in human plasma in a remarkably stable form and that they are not degraded at room temperature or after several freeze–thaw cycles. They went on to show by TaqMan qRT-PCR measurements that miRNA expression levels in plasma or serum were strongly correlated, indicating that both serum and plasma samples would be suitable for investigations of miRNAs as blood-based biomarkers. For their experiments, Mitchell et al. used a mouse prostate cancer xenograft model system in which a human prostate cancer cell line was implanted into mice. In this mouse model, miRNAs originating from the xenografts entered the circulation and could readily be detected in plasma from xenografted, but not from control mice. This concept was extended to the serum of human metastatic prostate cancer patients, in which one miRNA, miR-141, was highly overexpressed and could be used to distinguish patients with prostate cancer from healthy controls with 60% sensitivity and 100% specificity. As miR-141 is an epithelial-restricted miRNA, it seems possible that tumor-derived miRNAs could enter the circulation even when they originate from an epithelial cancer type, and that they can be detected in the circulation as a prostate cancer biomarker. The report thatmiR-141was overexpressed in the serum of prostate cancer patients contradicts somewhat the results of Lodes et al. who detected serum miRNAs in cancer using oligonucleotide microarrays [86]. Consistent with the results of Mitchell et al., they reported that serum miRNAs are upregulated in cancer patients as compared to normal donors. Comparing serum miRNA levels of late stages of prostate cancer patients and normal donors, they found that 15 miRNAs were upregulated in serum from prostate cancer patients compared to normal donor sera. However, they did not detect differential miR-141 expression in their studies. This discrepancy might have several reasons, for example, differences in the patient cohorts (treated versus untreated patients) or differences in the sensitivities of the methodologies used (microarray versus qRT-PCR analysis).

6. Circulating miRNAs associated with tumor development

The verdicts discussed in the earlier paragraph were based on contrasts of the miRNA serum levels between tumor patients and healthy controls. None of the described circulating miRNAs for the diagnosis of epithelial tumors showed correlations to histological subtypes, different stages or grades of tumor in further validation studies [87]-[90]. This suggests that the identified circulating miRNAs might be promising biomarkers for tumor detection but not necessarily appropriate for the prediction of the clinical courses of the diseases. In contrast, in tissue samples, miRNAs seem to be valuable markers to predict the clinical outcomes of tumor patients [91]-[93]. Brase et al [94] analyzed the miRNA profiles in sera of patients with highly aggressive compared to localized prostate cancer. Several circulating miRNAs were considerably higher abundant in patients with metastatic cancer. Two independent validation studies indicated that miRNA-141 and miRNA-375 were the most promising markers correlated with prostate tumor development. Recently, circulating miRNAs have also been reported to be correlated with clinicopathological variables (nodal and estrogen receptor status) in patients with breast cancer [39]. However, this study only demonstrated that circulating miRNAs are correlated with histopathological parameters, and not directly associated with patients' outcome. To evaluate the prognostic potential of the identified circulating miRNAs, larger retrospective validation studies integrating long-term follow-up data are required. For lung tumor, it was shown that serum miRNAs are promising prognostic biomarkers: Hu et al. demonstrated that circulating miRNAs can be used to predict the clinical outcomes of non-small-cell lung cancer (NSCLC) patients [95]. In their screening study, the authors compared the serum miRNA profiles of patients with long and short survival times using Solexa sequencing and validated the abundance of 13 selected miRNAs in 243 patients by qPCR. Four miRNAs (miRNA-486, miRNA-30d, miRNA-1, and miRNA-499) were confirmed to be associated with patient outcome. miRNA-1 was already described to be significantly down regulated in lung cancer tissue, thereby leading to decreased cell proliferation, migration and motility. miRNA-1 is supposed to target the MET oncogene as well as the gene HDAC4 [96]. Hu et al. also demonstrated that a combination of selected circulating miRNAs had a higher sensitivity than single biomarkers: Patients exhibiting large amounts of two or more high-risk miRNAs in the serum had a significantly increased probability of shorter survival times. Taken together, these results indicate a significant association of circulating miRNAs with the survival of NSCLC patients and therefore suggest that miRNAs are useful as prognostic markers.

7. Challenges and methodical advances to analyze circulating miRNAs

Despite the promising data supporting the potential value of miRNAs as biomarkers, many challenges remain. Due to the small amount of circulating miRNAs and the large amount of

proteins, miRNA isolation from serum samples is methodically challenging. To this end, numerous phenol/chloroform-based extraction protocols are available. Commercially available extraction kits without acid phase separation can also be used for the extraction of miRNA from body fluids. Circulating miRNAs can be extracted from both serum as well as plasma samples. Serum has recently been described to yield lower amounts of circulating miRNAs compared to plasma [97]. In addition, the effectiveness of serum samples has been questioned since the range of miRNAs from diverse samples can vary [97]. However, due to practical reasons, in the clinical practice, mainly serum samples are available. To this end, a good correlation was observed when the individual miRNA levels were compared between serum and plasma samples from the same patient donors [3]. Thus, both sample types seem to be suitable for the analysis of cell-free miRNAs. One of the main problems associated with circulating miRNA extraction and comparison of sample collectives is the quantification of the miRNA. The low abundance of miRNA in serum can hardly be determined using spectrophotometers. A robust and sensitive method for the analysis of serum miRNAs is their relative quantification by a stem-loop reverse transcription PCR (RT-PCR), which has been broadly used for the sensitive detection of low abundant circulating miRNAs [98] with high reproducibility. New methodicalies for serum-based miRNA analysis are emerging: For example, Lusi et al. designed a PCR- and label-free, sensitive detection method [99] based on an electrochemical sensor. After the hybrid formation of the miRNA with an inosine substitute, the oxidation of guanine generates an electrical signal, which can be quantified [99]. Microarray-based expression analysis is challenging since a large amount of RNA is needed for the analyses. Lodes et al. reported similar limits of detection for the analysis of circulating miRNAs for microarrays when compared to qPCR based methods [100]. Deep sequencing methodicalies have resulted in a steep increase of the rate of newly described microRNAs [101]. Since 2007, almost all newly recovered microRNAs were derived from deep sequencing analyses. The current release (miRBase 16) encompasses over 15.000 microRNA gene loci. The user can search for tissue- and stage specific expression, and compare own data with microRNA profiles in different diseases. First studies indicate disease-specific fingerprints in serum [2], [95]. Thus, large-scale miRNA sequencing appears to be very promising with respect to the identification of further biomarkers. Due to the described technical and quality variations, the strategy of raw data normalization is a critical issue. While some studies demonstrated a lack of significant differences between clinically defined entities [89], [17], miRNA-16 was also found to be highly abundant in the sera of prostate cancer patients [100]. Small non-coding RNAs are also commonly used. However, these have been reported to show limitations due to degradation in the blood stream [9], [102], [103]. Mitchell and colleagues reported a spike-in normalization approach to control for technical variances during the purification process. Three C. elegans miRNAs (without sequence similarity to known human miRNAs) were included in the purification procedure and used for data normalization [3], [11]. The mentioned challenges concerning extraction, quantification and data processing steps in miRNA analysis may lead to considerably variable results and clearly demonstrate the need for better standardization methods.

8. Discussion

Since tumor is fundamentally a dysregulation of gene expression, it is difficult to distinguish tumors which are morphologically similar but molecularly different by pathological assessment. For the earliest diagnosis, it is necessary to find noninvasive cancer biomarkers to monitor molecular differences in tumors, which may assist in the selection of the best possible treatment for individual cancer patients. Circulating miRNAs offer great anticipation for the diagnosis and prognosis, and possibly prediction, of tumor. However, there are still limitations to the technology and the recent study designs. In-line with the lessons learned from gene expression profiling, clinical associations of miRNA presence identified in small sample cohorts have to be verified in larger and independent studies, and the efforts to translate the findings into clinical practice have to be increased. Initial data of miRNA in several tumor entities were either based on literature search or on a limited number of miRNAs (Table 1). Worldwide screening studies monitoring a large set of human miRNAs are likely to lead to the discovery of better markers for specific diseases. In terms of biological or cellular functions, there is less known for any of the discovered serum miRNA. There are still controversial results concerning the relation of miRNA levels between tissue and corresponding serum: Several researchers suggested that tumor-associated miRNAs should be evaluated in the serum and in the tumor tissue [3, 88]. This is conceptually in line with the role of microvesicles in neoplastic progression [33]. However, in lung cancer, the let-7 family was shown to be associated with clinical outcome in tissue samples only, and was not detected in the serum [95]. Thus, circulating miRNAs may not always be directly associated with the changes occurring in tumor tissues but may also reflect indirect effects. On the other hand, deregulated circulating miRNAs have been reported to be significantly reduced in post-operative states [17], [102], [104]-[106]. Wang et al. concluded from their study that specific miRNAs are released into the blood stream after drug induced liver injury leading to a down regulation in the tissue [43]. In contrast to that, Tanaka et al. speculated that tumor cells rely on the specific intake of miRNAs from circulating microvesicles [87]. These contradictive assumptions clearly demonstrate a need for additional studies to elucidate the relation and function of tissue and serum miRNA expression levels. Furthermore, additional studies focusing on tumor specific microvesicles may provide insights into the biological roles of circulating miRNAs. Finally, it is unknown how soon miRNA changes appear in the serum, although some first results showed that miRNAs occur early in the blood stream during colon cancer development [102] and after drug induced liver injury [43]. Serum miRNAs-122 and 192 are detectable prior to the routine detection of liver injury using an alanine aminotransferase enzyme test [43]. So far, no study has analyzed the influence of age, health conditions or dynamical changes of the serum miRNA profile in different individuals. Therefore, the kinetics of circulating miRNAs should be analyzed in detail to unravel if infection diseases or lifestyle changes can lead to changes in the serum and to correct for these changes in future studies. Additionally it is unknown, if medical treatment leads to a change of the serum miRNA profiles. In-vitro studies revealed that specific microRNAs impact on drug sensitivity [107]. Thus, it is possible that personal treatments are reflected by serum microRNA profiles. This aspect is highly relevant, since the individual treatment

may influence the value of novel non-invasive miRNA biomarkers.

9. Conclusion

While I am just commencement to understand the function of circulating miRNAs in the serum, miRNAs in body fluids are presently widely investigated for their potential as non invasive diagnostic cancer markers. Tumor-specific circulating miRNAs may develop tumor diagnosis and prognosis, since numerous promising miRNAs have previously been depicted as noninvasive biomarkers for different tumor entities. However, larger sample sets including long-term clinical data are immediately required for future studies. In contrast, circulating miRNAs for the prophecy of drug responses have not been depicted so far. Isolation, quantification and normalization strategy have to be standardized before any of the novel miRNA biomarkers is applicable for clinical practice.

References

[1] World Health Organization (WHO), Cancer (WHO Fact Sheet No.297), 24/Oct/2006.

[2] Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, Zhang Y, Chen J, Guo X, et al, "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," Cell Res, (18), pp. 997-1006, 2008.

[3] Mitchell PS, Parkin RK, Kroh EM, Fritz BR, Wyman SK, Pogosova- Agadjanyan EL, Peterson A, Noteboom J, O'Briant KC, Allen A, et al, "Circulating microRNAs as stable blood-based markers for cancer detection," Proc Natl Acad Sci USA, (105), pp. 10513-10518, 2008.

[4] Lee RC, Feinbaum RL, Ambros V, "The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," Cell, (75), pp. 843–854, 1993.

[5] Kim, V. N., "Small RNAs: classification, biogenesis, and function, " Mol Cells, (19), pp. 1-15, 2005.

[6] Bartel, D. P, " MicroRNAs: genomics, biogenesis, mechanism, and function,".Cell, (116, pp. 281-97, 2004.

[7] Kim VN, Han J, Siomi MC, "Biogenesis of small RNAs in animals" Nat Rev Mol Cell Biol, (10**)**, pp. 126–139, 2009.

[8] Croce CM, "Causes and consequences of microRNA dysregulation in cancer," Nat Rev Genet, (10), pp. 704–714, 2009.

[9] Lawrie CH, Gal S, Dunlop HM, Pushkaran B, Liggins AP, Pulford K, Banham AH, Pezzella F, Boultwood J, Wainscoat JS, et al, "Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma," Br J Haematol, (141), pp. 672-675, 2008.

[10] Kroh EM, Parkin RK, Mitchell PS, Tewari M,"Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR)," Methods, (50), 4, pp**.** 298-301, 2010.

[11] Fichtlscherer S, De Rosa S, Fox H, Schwietz T, Fischer A, Liebetrau C, Weber M, Hamm CW, Roxe T, Muller-Ardogan M et al (2010). Circulating microRNAs in patients with coronary artery disease. *Circ Res*, **107**, 5, 677-684.

[12] Duttagupta R, Jiang R, Gollub J, Getts RC, Jones KW, "Impact of cellular miRNAs on circulating miRNA biomarker signatures.," PLoS One, (6), 6 e20769, 2011.

[13] Rupp AK, Rupp C, Keller S, Brase JC, Ehehalt R, Fogel M, Moldenhauer G, Marme F, Sultmann H, Altevogt P, "Loss of EpCAM expression in breast cancer derived serum exosomes: role of proteolytic cleavage," Gynecol Oncol, (122)**,** 2, pp. 437-446, 2011.

[14] Taylor DD, Gercel-Taylor C, "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer, " Gynecol Oncol, (110**)**, pp. 13-21, 2008.

[15] Gilad S, Meiri E, Yogev Y, Benjamin S, Lebanony D, Yerushalmi N, Benjamin H, Kushnir M, Cholakh H, Melamed N, et al, "Serum microRNAs are promising novel biomarkers," PLoS ONE, (3**)**, e3148, 2008.

[16] Ho AS, Huang X, Cao H, Christman-Skieller C, Bennewit K, Le QT, Koong AC, " Circulating miR-210 as a Novel Hypoxia Marker in Pancreatic Cancer," Transl Oncol, (3), pp. 109-113, 2010.

[17] Ji X, Takahashi R, Hiura Y, Hirokawa G, Fukushima Y, Iwai N, "Plasma miR-208 as a biomarker of myocardial injury," Clin Chem, (55), pp. 1944-1949, 2009.

[18] Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO, "Exosomemediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," Nat Cell Biol, (9), pp. 654-659, 2007.

[19] Caby MP, Lankar D, Vincendeau-Scherrer C, Raposo G, Bonnerot C, "Exosomal-like vesicles are present in human blood plasma," Int Immunol, (17), pp. 879-887, 2005.

[20] Van Niel G, Porto-Carreiro I, Simoes S, Raposo G, "Exosomes: a common pathway for a specialized function," J Biochem, (140), pp.13-21, 2006.

[21] Escola JM, Kleijmeer MJ, Stoorvogel W, Griffith JM, Yoshie O, Geuze HJ, "Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human Blymphocytes," J Biol Chem, (273**)**, pp. 20121-20127, 1998.

[22] Andre F, Schartz NE, Movassagh M, Flament C, Pautier P, Morice P, Pomel C, Lhomme C, Escudier B, Le Chevalier T, et al, "Malignant effusions and immunogenic tumour-derived exosomes," Lancet, (360), pp. 295-305, 2002.

[23] Valenti R, Huber V, Filipazzi P, Pilla L, Sovena G,Villa A, Corbelli A, Fais S, Parmiani G, Rivoltini L, "Human tumor-released microvesicles promote the differentiation of myeloid cells with transforming growth factor-betamediated suppressive activity on T lymphocytes," Cancer Res, (66**)**, pp. 9290-9298, 2006.

[24] Thery C, Regnault A, Garin J, Wolfers J, Zitvogel L, Ricciardi-Castagnoli P, Raposo G, Amigorena S, "Molecular characterization of dendritic cell- derived exosomes. Selective accumulation of the heat shock protein hsc73," J Cell Biol, (147), pp. 599-610, 1999.

[25] Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ, Geuze HJ, "B lymphocytes secrete antigen-presenting vesicles," J Exp Med, (183**)**, pp. 1161-1172, 1996.

[26] Fevrier B, Vilette D, Archer F, Loew D, Faigle W, Vidal M, Laude H, Raposo G, " Cells release prions in association with exosomes," Proc Natl Acad Sci USA (101**)**, pp. 9683-9688, 2004.

[27] Van Niel G, Raposo G, Candalh C, Boussac M, Hershberg R, Cerf- Bensussan N, Heyman M, "Intestinal epithelial cells secrete exosome-like Vesicles," Gastroenterology, (121), pp. 337-349, 2001.

[28] Volinia, S., G. A. Calin, C. G. Liu, S. Ambs, A. Cimmino, F. Petrocca, R. Visone, M. Iorio, C. Roldo, M. Ferracin, R. L. Prueitt, N. Yanaihara, G. Lanza, A. Scarpa, A. Vecchione, M. Negrini, C. C. Harris & C.M. Croce, "A microRNA expression signature of human solid tumors defines cancer gene targets," In Proceedings of the National Academy of Sciences of the United States of America (NAS), (103), pp. 2257-2261, 2006.

[29] Hunter MP, Ismail N, Zhang X, Aguda BD, Lee EJ, Yu L, Xiao T, Schafer J, Lee ML, Schmittgen TD, et al, "Detection of microRNA expression in human peripheral blood microvesicles," PLoS ONE, (3), e3694, 2008.

[30] Kosaka N, Iguchi H, Yoshioka Y, Takeshita F, Matsuki Y, Ochiya T, "Secretory Mechanisms and Intercellular Transfer of MicroRNAs in Living Cells," J Biol Chem, (285), pp. 17442-17452, 2010.

[31] Taylor DD, Doellgast GJ, "Quantization of peroxidase-antibody binding to membrane fragments using column chromatography," Anal Biochem, (98**)**, pp. 53-59, (1979).

[32] Rabinowits G, Gercel-Taylor C, Day JM, Taylor DD, Kloecker GH, "Exosomal microRNA a diagnostic marker for lung cancer," Clin Lung Cancer, (10), pp. 42-46, 2009.

[33] Skog J, Wurdinger T, van Rijn S, Meijer DH, Gainche L, Sena-Esteves M, Curry WT Jr, Carter BS, Krichevsky AM, Breakefield XO, "Glioblastoma microvesicles transport RNA and proteins that promote tumour growth and provide diagnostic biomarkers," Nat Cell Biol, (10), pp. 1470-1476, 2008.

[34] Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L, "Tumor released microvesicles as vehicles of immunosuppression," Cancer Res, (67), pp. 2912-2915, 2007.

[35] Baj-Krzyworzeka M, Szatanek R, Weglarczyk K, Baran J, Zembala M, "Tumour derived microvesicles modulate biological activity of human monocytes," Immunol Lett, (113), pp. 76-82, 2007.

[36] Heneghan HM, Miller N, Lowery AJ, Sweeney KJ, Newell J, Kerin MJ, "Circulating microRNAs as novel minimally invasive biomarkers for breast cancer," Ann Surg, (251), 3, pp. 499-505, 2010.

[37] Chin LJ, Slack FJ, "A truth serum for cancer-microRNAs have major potential as cancer biomarkers," Cell Res, (18), 10, pp. 983-984, 2008.

[38] Cortez MA, Calin GA, "MicroRNA identification in plasma and serum: a new tool to diagnose and monitor disease," Expert Opin Biol Ther, (9), pp. 703-711, 2009.

[39] Chim SS, Shing TK, Hung EC, Leung TY, Lau TK, Chiu RW, Lo YM, Detection and characterization of placental microRNAs in maternal plasma. Clin Chem, (54), pp. 482-490, 2008.

[40] Luo SS, Ishibashi O, Ishikawa G, Ishikawa T, Katayama A, Mishima T, Takizawa T, Shigihara T, Goto T, Izumi A, et a "Human villous trophoblasts express and secrete placenta-specific microRNAs into maternal circulation via exosomes," Biol Reprod, (81), pp. 717-729, 2009.

[41] Wang GK, Zhu JQ, Zhang JT, Li Q, Li Y, He J, Qin YW, Jing Q, "Circulating microRNA: a novel potential biomarker for early diagnosis of acute myocardial infarction in humans," Eur Heart J, (31), pp. 659-666, 2010.

[42] Adachi T, Nakanishi M, Otsuka Y, Nishimura K, Hirokawa G, Goto Y, Nonogi H, Iwai N, "Plasma MicroRNA 499 as a Biomarker of Acute Myocardial Infarction," Clin Chem, (56), pp. 1183-1185, 2010.

[43] Wang K, Zhang S, Marzolf B, Troisch P, Brightman A, Hu Z, Hood LE, Galas DJ, "Circulating microRNAs, potential biomarkers for drug-induced liver injury," Proc Natl Acad Sci USA, (106), pp. 4402-4407, 2009.

[44] Wang JF, Yu ML, Yu G, Bian JJ, Deng XM, Wan XJ, Zhu KM, "Serum miR- 146a and miR-223 as potential new biomarkers for sepsis," Biochem Biophys Res Commun," (394**)**, pp. 184-188, 2010.

[45] Vasilescu C, Rossi S, Shimizu M, Tudor S, Veronese A, Ferracin M, Nicoloso MS, Barbarotto E, Popa M, Stanciulea O, et al, "MicroRNA fingerprints identify miR-150 as a plasma prognostic marker in patients with sepsis," PLoS One, (4), e7405, 2009.

[46] Murata K, Yoshitomi H, Tanida S, Ishikawa M, Nishitani K, Ito H, Nakamura T, "Plasma and synovial fluid microRNAs as potential biomarkers of rheumatoid arthritis and osteoarthritis," Arthritis Res Ther, (12), R86, 2010.

[47] Calin GA, Croce CM, "MicroRNA signatures in human cancers," Nat Rev Cancer, (6), pp. 857-866, 2006.

[48] Esquela-Kerscher A, Slack FJ, "Oncomirs: MicroRNAs with a role in cancer," Nat Rev Cancer, (6), pp.259-269, 2006.

[49] Lu J, Getz G, Miska EA, "MicroRNA expression profiles classify human cancers," Nature, (435), pp. 834-838, 2005.

[50] Li J, Smyth P, Flavin R, et al, "Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells," BMC Biotechnol, (7), pp. 36, 2007.

[51] C.H. Lawrie, S. Gal, H.M. Dunlop, B. Pushkaran, A.P. Liggins, K. Pulford, A.H. Banham, F. Pezzella, J. Boultwood, J.S. Wainscoat, C.S. Hatton, A.L. Harris, "Detection of elevated levels of tumor-associated microRNAs in serum of patients with diffuse large B-cell lymphoma," Br. J. Haematol, (141), pp. 672–675, 2008.

[52] J. Skog, T. Würdinger, S. van Rijn, D.H. Meijer, L. Gainche, M. Sena-Esteves, W.T. Curry Jr, B.S. Carter, A.M. Krichevsky, X.O. Breakefield, "Glioblastoma microvesicles transport RNA and proteins that promote tumor growth and provide diagnostic biomarkers," Nat. Cell Biol, (10), pp. 1470–1476. 2008.

[53] M. Tanaka, K. Oikawa, M. Takanashi, M. Kudo, J. Ohyashiki, K. Ohyashiki, M. Kuroda, "Down-regulation of miR-92 in human plasma is a novel marker for acute leukemia patients," PLoS ONE, (4), e5532, 2009.

[54] Rodrigues VC, Moss SM, Tuomainen H, "Oral cancer in the UK: To screen or not to screen," Oral Oncology, (34), pp. 454–465, 1998.

[55] Parkin DM, Bray F, Ferlay J, Pisani P, "Global cancer statistics 2002," *CA* Cancer J Clin, (55), 2, pp. 74-108, 2005.

[56] Sano D, Myers JN, "Metastasis of squamous cell carcinoma of the oral tongue," Cancer Metastasis Rev, (26), 3-4, pp. 645-662, 2007.

[57] T.S. Wong, X.B. Liu, B.Y. Wong, R.W. Ng, A.P. Yuen, W.I. Wei, "Mature miR-184 as potential oncogenic microRNA of squamous cell carcinoma of tongue," Clin. Cancer Res, (14**)**, pp. 2588–2592, 2008.

[58] C.J. Liu, S.Y. Kao, H.F. Tu, M.M. Tsai, K.W. Chang, S.C. Lin, "Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer," Oral Dis, (16), pp. 360–364, 2010.

[59] S.C. Lin, C.J. Liu, J.A. Lin, W.F. Chiang, P.S. Hung, K.W. Chang, "miR-24 up-regulation in oral carcinoma: positive association from clinical and in vitro analysis," Oral Oncol, (46), pp. 204–208, 2010.

[60] Weir HK, Thun MJ, Hankey BF, et al, "Annual report to the nation on the status of cancer, 1975–2000, featuring the uses of surveillance data for cancer prevention and control.," J Natl Cancer Inst, (95), pp. 1276-1299, 2003.

[61] Society AC, "Cancer Facts & Figures 2008," American Cancer Society Atlanta, 2008.

[62] S. Taplin, L. Abraham, W.E. Barlow, J.J. Fenton, E.A. Berns, P.A. Carney, G.R. Cutter, E.A. Sickles, D. Carl, J.G. Elmore, "Mammography facility characteristics associated with interpretive accuracy of screening mammography," J. Natl Cancer Inst., (100), pp. 876–887, 2008.

[63] W. Zhu, W. Qin, U. Atasoy, E.R. Sauter, "Circulating microRNAs in breast cancer and healthy Subjects," BMC Research Notes, (2), 89, 2009.

[64] H.M. Heneghan, N. Miller, A.J. Lowery, K.J. Sweeney, J. Newell, M.J. Kerin, "Circulating microRNAs as novel minimally invasive biomarkers for breast cancer," Ann. Surg, (251), pp. 499–505,2010.

[65] G. Rabinowits, C. Gerçel-Taylor, J.M. Day, D.D. Taylor, G.H. Kloecke, "Exosomal microRNA: a diagnostic marker for lung cancer," Clin. Lung Cancer, (10), pp. 42–46, 2009.

[66] Z. Hu, X. Chen, Y. Zhao, T. Tian, G. Jin, Y. Shu, Y. Chen, L. Xu, K. Zen, C. Zhang, H. Shen, "Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer. J. Clin. Oncol, 28, 1721–1726, 2010.

[67] Shike M, Winawer SJ, Greenwald PH, et al. "Primary prevention of colorectal cancer," The WHO Collaborating Centre for the Prevention of Colorectal Cancer. Bull World Health Organ, (68), pp. 377-385, 1990.

[68] E.K. Ng, W.W. Chong, H. Jin, E.K. Lam, V.Y. Shin,J. Yu, T.C. Poon, S.S. Ng, J.J. Sung, "Differential expression of microRNAs in plasma of colorectal cancer patients: a potential marker for colorectal cancer screening," Gut, (58), 1375–1381, 2009.

[69] Z. Huang, D. Huang, S. Ni, Z. Peng, W. Sheng, X. Du, "Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer," Int. J. Cancer, (127), pp. 118–126, 2010.

[70] X. Chen, Y. Ba, L. Ma, X. Cai, Y. Yin, K. Wang, J. Guo, Y. Zhang, J. Chen, X. Guo, Q. Li, X. Li, W. Wang, Y. Zhang, J. Wang, X. Jiang, Y. Xiang, C. Xu, P. Zheng, J. Zhang, R. Li, H. Zhang, X. Shang, T. Gong, G. Ning, J. Wang, K. Zen, J. Zhang, C.Y. Zhang. "Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases," Cell Res, (10), pp. 997–1006, 2008.

[71] M. Tsujiura, D. Ichikawa, S. Komatsu, A. Shiozaki, H. Takeshita, T. Kosuga, H. Konishi, R. Morimura, K. Deguchi, H. Fujiwara, K. Okamoto, E. Otsuji, "Circulating microRNAs in plasma of patients with gastric cancers," Br. J. Cancer, (102), pp. 1174– 1179, 2010.

[72] S. Caldwell and S. H. Park, "The epidemiology of hepatocellular cancer: from the perspectives of public health problem to tumor biology," Journal of Gastroenterology, (44), 19, pp. 96–101, 2009.

[73] K. Hussain and H. B. El-Serag, "Epidemiology, screening, diagnosis and treatment of hepatocellular carcinoma," Minerva Gastroenterologica e Dietologica, (55), 2, pp. 123–138, 2009.

[74] P. Tandon and G. Garcia-Tsao, "Prognostic indicators in hepatocellular carcinoma: a systematic review of 72 studies," Liver International, (29), 4, pp. 502–510, 2009.

[75] H. B. El-Serag, "Hepatocellular carcinoma," New England Journal of Medicine, (365), 12, pp. 1118–1127, 2011.

[76] Y. Yamamoto, N. Kosaka, M. Tanaka, F. Koizumi, Y. Kanai, T. Mizutani, Y. Murakami, M. Kuroda, A. Miyajima, T. Kato, T. Ochiya, "MicroRNA- 500 as a potential diagnostic marker for hepatocellular Carcinoma," Biomarkers, (14), pp. 529–538, 2009.

[77] J. Wang, J. Chen, P. Chang, A. Leblanc, D. Li, J.L. Abbruzzesse, M.L. Frazier, A.M. Killary, S. Sen, "MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease," Cancer Prev. Res., (2), pp. 807–813, 2009.

[78] A.S. Ho, X. Huang, H. Cao, C. Christman-Skieller, K. Bennewith, Q.T. Le, A.C. Koong, "Circulating miR-210 as a novel hypoxia marker in pancreatic cancer," Transl. Oncol., (109), pp. 109–113, 2010.

[79] Jemal A, Siegel R, Ward E, et al, "Cancer Statistics 2008" CA Cancer J Clin, (58), pp. 71-96, 2008.

[80] Cannistra SA, "Cancer of the ovary,".N Engl J Med, (351), pp. 2519-2529, 2004.

[81] D.D. Taylor, C. Gerçel-Taylor, "MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer," Gynecol. Oncol., (110), pp. 13–21. 2008.

[82] K.E. Resnick, H. Alder, J.P. Hagan, D.L. Richardson, C.M. Croce, D.E. Cohn, "The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform" Gynecol. Oncol., (112), pp. 55–59,2009.

[83] Jemal A, Siegel R, Xu J, Ward E, Cancer statistics 2010, CA Cancer J Clin, (60), pp. 277-300, 2010.

[84] Nelen V, "Epidemiology of Prostate Cancer," In: Ramon J, Denis LJ, editors. Prostate Cancer, Berlin Heidelberg: Springer, pp. 1-8, 2007.

[85] P.S. Mitchell, R.K. Parkin, E.M. Kroh, B.R. Fritz, S.K. Wyman, E.L. Pogosova- Agadjanyan, A. Peterson, J. Noteboom, K.C. O'Briant, A. Allen, D.W. Lin, N. Urban, C. W. Drescher, B.S. Knudsen, D.L. Stirewalt, R. Gentleman, R.L. Vessella, P.S. Nelson, D.B. Martin, M. Tewari, "Circulating microRNAs as stable blood-based markers for cancer detection," Proc. Natl Acad. Sci. USA, (105), pp.10513–10518, 2008.

[86] M.J. Lodes, M. Caraballo, D. Suciu, S. Munro, A. Kumar, B. Anderson, "Detection of cancer with serum miRNAs on an oligonucleotide microarray," PLoS ONE, (4), e6229, 2009.

[87] Tanaka M, Oikawa K, Takanashi M, Kudo M, Ohyashiki J, Ohyashiki K, Kuroda M, "Down regulation of miR-92 in human plasma is a novel marker for acute leukemia patients," PLoS ONE, (4), e5532, 2009.

[88] Ng EK, Chong WW, Jin H, Lam EK, Shin VY, Yu J, Poon TC, Ng SS, Sung JJ, "Differential expression of microRNAs in plasma of patients with colorectal cancer: a potential marker for colorectal cancer screening," Gut, (58), 1375-1381, 2009.

[89] Wang J, Chen J, Chang P, LeBlanc A, Li D, Abbruzzesse JL, Frazier ML, Killary AM, Sen S, "MicroRNAs in plasma of pancreatic ductal adenocarcinoma patients as novel blood-based biomarkers of disease," Cancer Prev Res, (2), pp. 807-813, 2009.

[90] Resnick KE, Alder H, Hagan JP, Richardson DL, Croce CM, Cohn DE, "The detection of differentially expressed microRNAs from the serum of ovarian cancer patients using a novel real-time PCR platform," Gynecol Oncol, (112), pp. 55-59. 2009.

[91] Yu SL, Chen HY, Chang GC, Chen CY, Chen HW, Singh S, Cheng CL, Yu CJ, Lee YC, Chen HS, et al, "MicroRNA signature predicts survival and relapse in lung cancer," Cancer Cell, (13), pp. 48-57, 2008.

[92] Schetter AJ, Leung SY, Sohn JJ, Zanetti KA, Bowman ED, Yanaihara N, Yuen ST, Chan TL, Kwong DL, Au GK, et al, "MicroRNA expression profile associated with prognosis and therapeutic outcome in colon adenocarcinoma," JAMA, (299), pp. 425- 436, 2008.

[93] Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, Iorio MV, Visone R, Sever NI, Fabbri M, et al , "A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia," N Engl J Med, (353), 1793-1801, 2005.

[94] Brase JC, Johannes M, Schlomm T, Falth M, Haese A, Steuber T, Beissbarth T, Kuner R, Sultmann H, "Circulating miRNAs are correlated with tumor progression in prostate cancer," Int J Cancer, Epub. 2010.

[95] Hu Z, Chen X, Zhao Y, Tian T, Jin G, Shu Y, Chen Y, Xu L, Zen K, Zhang C, Shen H, "Serum microRNA signatures identified in a genome-wide serum microRNA expression profiling predict survival of non-small-cell lung cancer," J Clin Oncol, (28), 1721-1726, 2010.

[96] Nasser MW, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, Wang B, Suster S, Jacob ST, Ghoshal K , "Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1," J Biol Chem, (283), 33394-33405, 2008

[97] Heneghan HM, Miller N, Kerin MJ, "Circulating miRNA Signatures: Promising Prognostic Tools for Cancer," J Clin Oncol, (28), pp. e573-574, 2010.

[98] Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, et al, "Real-time quantification of microRNAs by stem-loop RT-PCR," Nucleic Acids Res, (33), pp. e179, 2005.

[99] Lusi EA, Passamano M, Guarascio P, Scarpa A, Schiavo L, " Innovative electrochemical approach for an early detection of microRNAs," *Anal* Chem, (81), pp. 2819-2822, 2009.

[100] Lodes MJ, Caraballo M, Suciu D, Munro S, Kumar A, Anderson B, "Detection of cancer with serum miRNAs on an oligonucleotide microarray," PLoS One, (4), pp. e6229, 2009.

[101] Kozomara A, Griffiths-Jones S, " miRBase: integrating microRNA annotation and deep- sequencing data," Nucleic Acids Res, Epub, 2010.

[102] Huang Z, Huang D, Ni S, Peng Z, Sheng W, Du X, "Plasma microRNAs are promising novel biomarkers for early detection of colorectal cancer," Int J Cancer, (127), pp. 118-126, 2010.

[103] Zhu W, Qin W, Atasoy U, Sauter ER, "Circulating microRNAs in breast cancer and healthy subjects," BMC Res Notes, (2), pp. 89, 2009.

[104] Liu CJ, Kao SY, Tu HF, Tsai MM, Chang KW, Lin SC, " Increase of microRNA miR-31 level in plasma could be a potential marker of oral cancer," Oral Dis, (16), pp. 360-364, 2010.

[105] Wong TS, Liu XB, Wong BY, Ng RW, Yuen AP, Wei WI, Mature miR-184 as Potential Oncogenic microRNA of Squamous Cell Carcinoma of Tongue," Clin Cancer Res, (14), pp 2588- 2592, 2008.

[106] Tsujiura M, Ichikawa D, Komatsu S, Shiozaki A, Takeshita H, Kosuga T, Konishi H, Morimura R, Deguchi K, Fujiwara H, et al, "Circulating microRNAs in plasma of patients with gastric cancers," Br J Cancer, (102), pp. 1174-1179, 2010.

[107] Rao X, Di Leva G, Li M, Fang F, Devlin C, Hartman-Frey C, Burow ME, Ivan M, Croce CM, Nephew KP, "MicroRNA-221/222 confers breast cancer fulvestrant resistance by regulating multiple signaling pathways," Oncogene, Epub, 2010.