Early detection of cancer is crucial for its ultimate control and the prevention of malignant progression. In Japan, a nationwide project was conducted between 2014 and 2019 to develop novel cancer detection tools using serum microRNAs (miRNAs). Using the National Cancer Center Biobank, we collected more than 10,000 serum samples from patients with malignant diseases, including rare cancers such as ovarian cancer, gliomas, and sarcomas. Subsequently, comprehensive miRNA microarray analyses were performed for all samples. This serum miRNA database provides insights regarding miRNA biomarker candidates for each cancer type. Here, we summarize the major achievements of this national project. Notably, although circulating miRNAs packaged in extracellular vesicles are thought to be a cell-to-cell communication tool, the functional characteristics of the miRNAs listed in the project are still unknown. We hope that our findings will help elucidate the biological functions of circulating miRNAs.

**Keywords:** miRNA, extracellular vesicle, biomarker, cancer, serum

**Introduction**

Cancer has been the most common cause of death in Japan since 1981. According to the Japanese Ministry of Health, Labour, and Welfare, 27.4% of all deaths were attributable to cancer in Japan in 2018. Effectively treating and curing cancer is directly dependent on the ability to detect cancers as early as possible. However, population-based cancer screening is conducted for only five cancer types—gastric, colorectal, lung, breast, and cervical cancers—because of insufficient evidence supporting the benefits of screening for other cancer types. Furthermore, cancer screening rates are much lower in Japan than in other developed countries. To overcome this situation, less-invasive, highly-accurate, novel screening technologies that cover all malignant diseases are warranted worldwide. In this context, circulating microRNAs (miRNAs) have recently received significant attention as one of the most promising next-generation biomarkers. Here, we summarize our contribution to studies of circulating miRNAs and discuss the future direction of research.

**Functional Importance of Extracellular miRNAs**

miRNAs are short, non-coding RNA molecules that are 17–25 nucleotides in length. They modulate target gene expression at the post-translational level by guiding the RNA-induced silencing complex to miRNA target sites in the 3′ untranslated region of messenger RNAs (mRNAs), leading to mRNA degradation or the inhibition of translation. Dysregulated miRNAs can contribute to various pathophysiologicals, including cancer initiation and development. miRNAs are secreted by cells and exist stably in body fluids within extracellular vesicles (EVs) or bound to proteins or lipids. EVs are a heterogeneous collection of membrane-bound...
Matsuzaki, J and Ochiya T: Circulating MicroRNAs carriers with complex cargos, including proteins, lipids, mRNAs, and miRNAs. In general, EVs are thought to be produced either by the release of intraluminal vesicles contained inside the endosome-derived multivesicular body by fusion with the plasma membrane or by direct assembly and budding from the plasma membrane (Fig. 1). Exosomes are enriched in smaller EV components (50–150 nm in diameter). Although the release of EVs was previously thought only to be a mechanism to discard nonfunctional cellular components, increasing evidence suggests that EVs play a key role in communication with neighboring and distant cells. EVs bear surface molecules that facilitate targeting by recipient cells. Once attached to a recipient cell, EVs can induce signaling via receptor–ligand interaction, be internalized by endocytosis and/or phagocytosis, or even fuse with the target cell’s membrane to deliver their contents into the cytosol, thereby modifying the physiological state of the recipient cell.

A large body of in vitro evidence demonstrates that miRNA secreted in EVs can be functionally delivered to target cells, resulting in direct modulation of their mRNA targets. Within the context of cancer biology, the altered miRNA composition within EVs can promote tumor progression and metastasis by contributing to the cancer microenvironment. For example, exosomal miR-181c derived from breast cancer cells promotes breakdown of the blood–brain barrier through abnormal localization of actin via downregulation of its target 3-phosphoinositide-dependent protein kinase-1 in endothelial cells. An exosomal miRNA-mediated intercellular communication mechanism also contributes to the development of chronic obstructive pulmonary disease. Cigarette smoke extract induces upregulation of exosomal miR-210 in primary human bronchial epithelial cells. Subsequently, exosomal miR-210 regulates autophagy processes by targeting ATG7 and promotes myofibroblast differentiation in lung fibroblasts.

It is therefore evident that miRNAs are actively secreted from cells and play crucial roles in intercellular communication. This fact explains why circulating miRNA profiles reflect a subject’s physiological and pathological...
status and are promising biomarkers for various disease states.

Parameters for Assessing the Performance of Diagnostic Biomarkers

The usefulness and interpretation of clinical biomarkers are highly dependent on their diagnostic performance. The performance of a discriminant between two groups, such as the presence and the absence of a disease, is assessed by a receiver operating characteristic (ROC) curve (Fig. 2).

- It is a plot of the false-positive rate (x-axis) versus the true-positive rate (y-axis) for all possible threshold values of the test, and the area under the ROC curve (AUC) represents the discriminability of the two groups by the test. Traditionally, the AUC (range from 0.5 to 1) can be interpreted as follows: 0.90–1, excellent; 0.80–0.90, good; 0.70–0.80, fair; 0.60–0.70, poor; and 0.50–0.60, fail. Based on the optimal cut-off level of the test set by a ROC curve, the true-positive rate (sensitivity) and the true-negative rate (specificity) are calculated. There is a trade-off between sensitivity and specificity (range from 0 to 1). When the sample size is large enough, analyzed samples should be divided into a training set to establish a cut-off value and a validation set for calculating sensitivity and specificity.

Biomarkers for Cancer Detection

Over the past decade, miRNA analytical methods have rapidly progressed, and next-generation sequencing (NGS) is becoming a standard of comprehensive miRNA expression analysis. Before the NGS era, only microarray analysis was available to comprehensively analyze miRNA profiles. Ogata-Kawata et al. performed microarray analyses of miRNAs in exosome-enriched fractions of serum samples from 88 patients with primary colorectal cancer (CRC) and 11 healthy controls using the Agilent miRNA microarray platform. The serum exosomal levels of seven miRNAs (let-7a, miR-1229, miR-1246, miR-150, miR-21, miR-223, and miR-23a) were significantly higher in patients with primary CRC, even in those with early-stage disease, than in healthy controls, and were significantly down-regulated after surgical resection of the tumors. These miRNAs were also secreted at significantly higher levels by colon cancer cell lines than by a normal colon-derived cell line. The expression levels of selected miRNAs were also validated by quantitative reverse transcription polymerase chain reaction analyses of an independent set of 13 patients with CRC. Kojima et al. skipped the EV isolation process and extracted total RNAs from 571 serum samples obtained from healthy patients; patients with pancreatic, biliary-tract, or other digestive cancers; and patients with non-malignant abnormalities in the pancreas or biliary tract. Subsequently, comprehensive miRNA expression profiles were examined using the highly sensitive 3D-Gene microarray platform (Toray Industries, Inc.). Then, they developed a diagnostic index using a combination of eight miRNAs (miR-6075, miR-4294, miR-6880-5p, miR-6799-5p, miR-125a-3p, miR-4530, miR-6836-3p, and miR-4476) and achieved a sensitivity, specificity, and AUC of 0.80, 0.98, and 0.95, respectively. In the same test cohort, the sensitivity and specificity of CA19-9 and CEA was 0.66 and 0.40, and 0.93 and 0.89, respectively. These striking results proved the concept that serum miRNA profiling can be a powerful tool to detect cancers.

In 2014, a large-scale national project in Japan—Development and Diagnostic Technology for Detection of miRNA in Body Fluids—was launched; it was supported by the New Energy and Industrial Technology Development Organization and the Japan Agency for Medical Research and Development. This project aimed to facilitate early detection of 13 types of malignant diseases—breast, lung, gastric, colorectal, esophagus, liver, pancreatic, biliary tract, prostate, bladder, and ovarian cancers; sarcomas; and gliomas—using serum miRNAs. Through this project, we accumulated evidence regarding the utility of serum miRNA testing (Fig. 3). Although not all the achievements of the project have yet been published, we present here a short summary of some of the published results.
Matsuzaki, J and Ochiya T: Circulating MicroRNAs

To establish a diagnostic model for ESCC, serum miRNA expression profiles of patients with ESCC ($n = 566$) and control patients without cancer ($n = 4965$) were retrospectively analyzed. Patients histologically diagnosed with ESCC who had not received prior therapy and had no past or concurrent cancer other than ESCC were enrolled from the National Cancer Center Hospital in Tokyo, Japan. Control samples were collected from the National Cancer Center Biobank, the Biobank of the National Center for Geriatrics and Gerontology, and the general population undergoing routine health examinations between October 2010 and November 2015. Serum samples were randomly divided into discovery and validation sets.

Figure 3. Serum miRNAs able to detect cancer. The miRNAs shown here were identified in the Development and Diagnostic Technology for Detection of miRNA in Body Fluids project. The miRNAs used to detect pancreatobiliary/colorectal cancer were identified before the project.
The expression levels of 2588 miRNAs were assessed in each sample using the 3D-Gene microarray. Using Fisher linear discriminant analysis with a greedy algorithm, a diagnostic index using six miRNAs (miR-8073, miR-6820-5p, miR-6794-5p, miR-3196, miR-744-5p, and miR-6799-5p) was developed. In the validation set, the area under the receiver operating characteristic curve for the diagnostic index was 1.00, with a sensitivity and specificity of 0.96 and 0.98, respectively.\(^{19}\)

The incidence of EAC has dramatically increased in Western countries, but it is still rare in Japan. Therefore, instead of analyzing human samples, we comprehensively analyzed tissue and serum miRNA expression profiles of an EAC mouse model (L2-interleukin-1β mice)\(^{20}\) using 3D-Gene. In mice, we identified 20 upregulated miRNAs and 44 downregulated miRNAs in tissues and sera during the development of Barrett’s esophagus (BE), a precursor lesion of EAC. To validate the data from mice, a published dataset of human plasma miRNAs from eight patients with EAC, eight with BE, and six healthy controls was used.\(^{21}\) Compared with the plasma of patients with BE, 2 of 20 miRNAs (miR-128-3p and miR-328-3p) were upregulated and 5 of 44 miRNAs (miR-143-3p, miR-144-3p, miR-15a-5p, miR-1-3p and miR-133b) were downregulated in the plasma of patients with EAC. A prediction index calculated using the abovementioned seven miRNAs could discriminate between patients with EAC and those without EAC with an AUC, sensitivity, and specificity of 0.91, 1.00, and 0.75, respectively.\(^{22}\)

**Breast Cancer**

Breast cancer is one of the most common cancers among Japanese women. We comprehensively evaluated the serum miRNA expression profiles of 1280 serum samples of patients with breast cancer stored in the National Cancer Center Biobank. We also analyzed 2836 serum samples from non-cancer controls, 451 serum samples from patients with other types of cancers, and 63 serum samples from non-cancer controls, 451 serum samples from patients with other types of cancers, and 63 serum samples from non-cancer controls. A combination of five miRNAs (miR-1246, miR-1307-3p, miR-4634, miR-6861-5p, and miR-6875-5p) had a sensitivity and specificity of 0.973 and 0.829, respectively, for breast cancer detection in the test cohort.\(^{23}\)

**Lung Cancer**

An accurate early screening method for lung cancer would be a powerful tool for decreasing lung cancer-related mortality. We examined the miRNA profiles from 3744 serum samples obtained from 1566 patients with resectable lung cancer and 2178 participants with no known cancer. We created a diagnostic model for lung cancer based on the combined expression levels of two miRNAs (miR-1268b and miR-6075) in the discovery set (208 patients with lung cancer and 208 participants with no cancer). The model had a sensitivity of 0.99 and a specificity of 0.99 in the validation set (1358 patients with lung cancer and 1970 participants with no known cancer). The diagnostic model exhibited high sensitivity regardless of the histological type and pathological TNM cancer stage.\(^{24}\)

**Hepatocellular Carcinoma**

Hepatocellular carcinoma (HCC) is the fourth leading cause of cancer deaths worldwide.\(^ {18}\) We analyzed serum samples from 345 patients with HCC, 46 patients with chronic hepatitis, 93 patients with liver cirrhosis, and 1033 healthy individuals using a miRNA microarray. We selected 52 miRNAs whose expressions were affected by the disease progression status, established a diagnostic model that used a combination of eight miRNAs (miR-320b, miR-663a, miR-4448, miR-4651, miR-4749-5p, miR-6724-5p, miR-6877-5p, and miR-6885-5p) in the discovery set, and tested the model in the validation set. The diagnostic values for discriminating cancer from HCC at-risk control samples were as follows: AUC, 0.99; sensitivity, 0.977; and specificity, 0.947. With this model, 98% of stage I HCC cases were detected, which was a much better rate than using α-fetoprotein as a biomarker.\(^ {25}\)

**Ovarian Cancer**

Among gynecologic malignancies, ovarian cancer has the highest mortality rate because the disease is often detected late in its progression: approximately 75% of patients present with stage III or IV ovarian cancer with widespread metastasis in the peritoneal cavity. We analyzed the miRNA profiles of 4046 serum samples, including 428 from patients with ovarian tumors. A diagnostic model based on the expression levels of ten miRNAs (miR-320a, miR-665, miR-3184-5p, miR-6717-5p, miR-4459, miR-6076, miR-3195, miR-1275, miR-3185, and miR-4640-3p) was constructed in the discovery set. Validation in an independent cohort revealed that the model was very accurate (sensitivity, 0.99 and specificity, 1.00), and the diagnostic accuracy was maintained even for early-stage ovarian cancers. Furthermore, we investigated whether the serum miRNA profile could discriminate ovarian cancers from borderline or benign ovarian tumors. Distinguishing benign tumors from malignant cancers is a major concern for gynecologists, and a less-invasive diagnostic method would be of great clinical value. However, we found it difficult to discriminate between ovarian cancer and borderline ovarian tumors. The sample sizes of borderline or benign ovarian tumors were relatively small; consequently, further large-scale validation studies are needed.\(^ {26}\)
Prostate Cancer

The high false-positive rates of prostate-specific antigen (PSA) may lead to unnecessary prostate biopsies. Therefore, the United States Preventive Services Task Force recommends that decisions regarding PSA-based screening for prostate cancer in men aged 55–69 years be made with caution, and that men aged ≥70 years should not undergo PSA screening. To overcome this problem, we investigated the potential of serum miRNAs as an accurate diagnostic tool in patients with suspected prostate cancer. Serum samples of 809 patients with prostate cancer, 241 patients with negative prostate biopsies, and 500 patients with other cancer types were obtained from the National Cancer Center, Japan. Forty-one healthy control samples were obtained from two hospitals in Japan. In the discovery set, 18 candidate miRNAs were identified. A robust diagnostic model was constructed using a combination of two miRNAs (miR-17-3p and miR-1185–2-3p) in the training set. A high diagnostic performance, with a sensitivity of 0.90 and a specificity of 0.90, was achieved in the validation set regardless of the Gleason score or the clinical TNM stage.27

Bladder Cancer

The major problem in bladder cancer is primarily the high recurrence rate after treatment. We performed miRNA profiling of 392 serum samples of bladder cancer patients, with 100 non-cancer samples and 480 samples of other cancer types as controls. We identified a specific miRNA, miR-6087, for diagnosing bladder cancer. We also found that a combination of seven miRNAs (miR-6087, miR-6724-5p, miR-3960, miR-1343-5p, miR-1185–1-3p, miR-6831-5p, and miR-4695-5p) could discriminate bladder cancer from non-cancerous tumors and other types of tumors with the highest accuracy (AUC: 0.97; sensitivity: 0.95; specificity: 0.87). The diagnostic accuracy was high, regardless of the stage and grade of bladder cancer.28

Glioma

The blood-based diagnosis of brain tumors is attractive considering the high anatomical invasiveness of pathological diagnosis. First, we designed a diagnostic index to discriminate between patients with diffuse glioma (n =100) and patients with no brain disease (n =200). The Glioma Index was constructed using three miRNAs (miR-4763-3p, miR-1915-3p, and miR-3679-5p). The AUC, sensitivity, and specificity were 0.99 (95% confidence interval [CI], 0.99–1.00), 0.95 (95% CI, 0.89–1.00), and 0.97 (95% CI, 0.93–1.00), respectively, in the validation set. The Glioma Index classified 93% of primary central nervous system lymphomas (PCNSL) and 89% of metastatic brain tumors (Meta) as positive, indicating that this index can also detect brain tumors other than glioma. We then tried to discriminate among glioblastoma (GBM), PCNSL, and Meta. The 3-Tumor Index was constructed using 48 miRNAs and achieved an accuracy of 0.80, positively detecting 94% of GBM, 80% of Meta, and 50% of PCNSL in the validation set. Consequently, although complicated calculations using many miRNA expression levels were required, serum miRNA combinations could be utilized to assess brain tumor histology.29

Sarcomas

Because of their rarity and diversity, sarcomas are difficult to diagnose, especially for primary care physicians. We investigated serum miRNA profiles from 1002 patients with bone and soft tissue tumors (representing more than 43 histological subtypes), including sarcomas, intermediate tumors, and benign tumors, to determine whether serum miRNA profiles could be used to specifically detect sarcomas. Circulating serum miRNA profiles in sarcoma patients were clearly distinct from those in patients with other types of bone and soft tissue tumors. Using the serum levels of seven miRNAs (miR-4736, miR-6836-3p, miR-4281, miR-762, miR-658, miR-4649-5p, miR-4665-3p), we developed a diagnostic index that could distinguish sarcoma patients from those with benign tumors and healthy controls with 0.90 sensitivity and 0.95 specificity, regardless of the histological subtype.30

Uterine Sarcoma

Uterine leiomyosarcoma (ULMS) is the major subtype of uterine sarcoma (US) and contributes significantly to uterine cancer deaths. Although the preoperative diagnosis of US remains challenging, frequent application of laparoscopic surgery for benign uterine leiomyomas (ULM) requires precise exclusion of US. Therefore, we tried to identify diagnostic biomarkers for distinguishing US from ULM by focusing on circulating miRNAs. Although the sample sizes were quite small, (ULMS, 6; ULM, 18; endometrial stromal sarcoma, 2; adenosarcoma, 2; uterine smooth muscle tumor of uncertain malignant potential, 1), we developed an optimal model consisting of two miRNAs (miR-1246 and miR-191-5p), with an AUC for identifying ULMS of 0.97 (95% CI, 0.91–1.00). In contrast, serum lactate dehydrogenase had an AUC of only 0.64 (95% CI, 0.34–0.94).31

Future Directions

Through the Development and Diagnostic Technology for Detection of miRNA in Body Fluids project, we found that serum miRNA analysis was highly accurate in discriminating between cancer and non-cancer samples. This fact has boosted enthusiasm worldwide for the development of novel cancer detection systems using miR-
NAs. In parallel, studies of circulating cell-free DNA biomarkers are also actively progressing. Furthermore, urinary miRNAs could also serve as biomarkers for several types of cancers. As a result, nucleic acid-based non-invasive testing could drastically change the landscape of cancer diagnostic strategies in the future.

The utility of circulating miRNAs is not limited to cancer detection. In breast cancer patients, serum miRNAs can be useful predictors of initial lymph node metastasis, distant metastasis after treatment, and the side effects of treatment. In HCC patients, we also found that serum miR-1246 can help to predict early tumor recurrence within 12 months of hepatectomy. The prediction of the treatment response to immune checkpoint inhibitors is also attractive, considering the high cost of such treatment. In patients with ESCC, we conducted a pilot study using serum samples from the phase II trial of nivolumab, a programmed cell death protein 1 inhibitor (JapicCTI-No. 142422). We identified miRNAs associated with the response to nivolumab, including one detected in the serum before treatment (miR-1233-5p; AUC =0.895) and three present after treatment (miR-6885-5p, miR-4698, and miR-128–2–5p; AUC =0.93, 0.97, and 0.93, respectively). In addition, we also suggested the possibility of predicting the risk of Alzheimer’s disease and cerebrovascular disorders before the onset of stroke using serum miRNAs. Taken together, these findings suggest that circulating miRNA analyzing technologies could play a significant role in various aspects of clinical decision making.

The most important challenge for the future is to elucidate the biological characteristics of circulating miRNAs (Fig. 4). We believe that changes in the serum miRNA profiles of cancer patients cannot be fully explained by the abnormal expression of miRNAs in cancer cells. Because the downstream signal pathways of miRNAs vary among cell types, it is meaningless to speculate on the functions of miRNAs without identifying the donor cells or recipient cells of circulating miRNAs. Biological confirmation of why these miRNAs are dysregulated in the corresponding disease setting would make it easier to understand the reasons for pseudo-positive and pseudo-negative miRNA tests in clinical practice. We hope that our results will contribute to further research on the biological aspects of circulating miRNAs.

Based on our data and experience, some “prototype” products might be released within a few years. Companies collaborating on the national project, such as Toray

![Figure 4. Mysteries in the biological aspects of circulating miRNA. Utilizing miRNA biomarkers can be challenging without understanding the biological characteristics of circulating miRNAs. Here, we summarized the main issues that remain to be elucidated.](image-url)
Industries, Toshiba, and Arkray, are trying to enter the market. However, we believe that it will take more time before miRNA testing can inform our daily clinical practice. Furthermore, a better understanding of the biological aspects of circulating miRNAs is required before these products can be made available for commercial use. Nevertheless, the hypothesis that circulating miRNAs can be effective cancer biomarker was well validated using a large number of clinical samples. This fact will further accelerate the study of circulating miRNAs.

Conflicts of Interest

There are no potential conflicts of interest relevant to this work.

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