Liquid biopsy of gastric cancer patients: Circulating tumor cells and cell-free nucleic acids

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer...
and the second leading cause of cancer-related death in the world\cite{9}. Although recent improvements in diagnostic techniques and peri-operative management have resulted in an increase in the early detection of GC and a decrease in its mortality in the past decades, a total of 986600 new GC cases and 738000 deaths are estimated to have occurred in 2008 worldwide\cite{10}. Several factors seem to restrict diagnostic and therapeutic strategy for treatment of GC and, consequently, to incur the insufficient survival rate: (1) a lack of satisfactory diagnostic assays for early detection of GC; (2) an absence of valuable prognostic indicators; (3) the insufficient effectiveness of current treatments including surgery and chemotherapy for GC patients with advanced stages; and (4) poorly understood mechanisms of tumor progression and resistance to treatments, and a consequent deficiency of targeted therapy. Therefore, the importance of developing useful diagnostic and monitoring tools should be emphasized to improve the clinical outcome of patients with GC.

In the past few decades, numerous studies have demonstrated the potential utility of blood-based biomarkers such as circulating tumor cells (CTCs) and cell-free nucleic acids (cfNAs)\cite{11,12,13}. These promising markers are considered to possess great potential and could facilitate therapeutic strategies for cancer including the following: early detection of diseases, predication of prognostic outcome, monitoring of tumor dynamics and development of novel targeted treatments.

Generally, tumor-linked genetic alterations are investigated using tissue samples from surgical or biopsy specimens. These procedures cannot be conducted routinely owing to their invasive nature especially in recurrent and/or metastatic cases with anatomical and/or clinical difficulties. Moreover, a result acquired from a single biopsy can provide only spatiotemporally restricted information and may fail to reflect its heterogeneity and inconsistent tumor characteristics. Detecting CTCs and cfNAs could serve as a “liquid biopsy” for cancer patients, which would be less invasive compared to surgical or endoscopic biopsy and allow us to have repeated samplings and to track the current status of tumor characteristics, such as therapeutic efficiency and resistance. From these viewpoints, the concept of “liquid biopsy” may lead to a better understanding of the genetic landscape in both primary and metastatic lesions as well as the opportunity for tracing genomic evolution.

In this article, we review the historical backgrounds, characterizations and recent developments of both CTCs and cfNAs in cancer research including GC and discuss future perspectives.

**BIOLOGY AND DETECTION OF CTCs**

In 1869, Ashworth reported the presence of CTCs for the first time in a case of a metastatic cancer patient, in whom cells similar to those in the primary tumors were found in the blood at autopsy\cite{14}. Since then, various studies have demonstrated the identification and characterization of CTCs in peripheral blood of patients with various malignancies, validating Ashworth’s previous remarks. Generally, CTCs are considered to appear at very low concentrations in the peripheral blood of cancer patients, usually a single tumor cell in a background of millions of blood cells\cite{15,16}. Thus, the accurate detection of CTCs with sufficient sensitivity and specificity has been a major technical challenge for researchers (Figure 1).

**Techniques for the isolation and enrichment of CTCs**

The approaches of CTC isolation/enrichment can be mainly categorized into two groups: (1) physical methods; and (2) biological methods. Isolation based on physical properties does not require the immunological labeling of CTCs because it depends on the characteristics of CTCs, such as size, density, electric charge, migratory capacity and deformability. These methods include density gradient centrifugation, filtration, and dielectrophoresis. Several filtration-based approaches have been developed based on the concept that the majority of CTCs derived from epithelial cancers are generally larger in diameter than other blood cells\cite{17,18}. However, significant variations in cell size within an individual patient as well as in different types of tumor cells have been reported\cite{19,20,21}. Therefore, new approaches using multiple filters have been investigated to resolve those issues and achieve the accurate enrichment of CTCs\cite{22,23}. While those new approaches are likely to possess great promise in isolating CTCs, further validation studies should be conducted to verify their significance.

Biological methods are another popular approach for the isolation of CTCs, which rely on immunological antibody-based capture of CTCs. In general, this assay involves positive selection with antibodies against tumor-associated antigens, such as epithelial cell adhesion (EpCAM) and cytokeratins (CKs), as well as negative selection with antibodies against the common leukocyte antigen CD45. In particular, EpCAM has been demonstrated to be over expressed and to function as an oncoene in human epithelial cancers including GC\cite{24,25,26}. Among several technologies based on antibody-based isolation, the CellSearch system (Veridex) is the most widely used separation system. In this platform, immunomagnetic beads coated with anti-EpCAM antibodies capture CTCs, followed by immunostaining with both positive markers, which are CK8/18/19 for cytoplasmic epithelial markers and 4',6-diamidino-2-phenylindole hydrochloride for nucleic acids, and a negative marker, leukocyte-specific marker CD45. Accumulating studies have demonstrated the usefulness of the CellSearch system as diagnostic and prognostic indicator in patients with metastatic disease. To date, it is the only technology that has been approved by the United States Food and Drug Administration for the detection of CTCs in the peripheral blood of patients with metastatic breast, prostate and colon cancers\cite{27,28,29}.

CTCs are generally thought to be quite heterogeneous in both phenotype and genotype, and only a few cells with malignant features could develop into metastatic...
During the journey toward the development of a metastatic lesion, some CTCs might undergo the epithelial-to-mesenchymal transition (EMT), which is characterized by decreased expression of epithelial markers and the acquisition of mesenchymal features. The EMT has been proposed to be frequently related with cancer aggressiveness and might increase the ability of tumor cells to migrate. Although the identification of EMT-like cancer cells in the bloodstream and its relevance to cancer dissemination is currently under evaluation, assays targeting only epithelial cells may miss the most invasive and potentially significant subpopulation with respect to cancer progression. Therefore, alternative enrichment approaches with different epithelial antigens or negative selection methods aimed to avoid the biased selection of CTC population might be advantageous.

Tumor-specific markers, such as human epidermal growth factor 2 (HER2), prostate-specific antigen (PSA), mucin-1/2 (MUC1/2) and carcinoembryonic antigen (CEA), have also been implemented to capture CTCs more specifically and adapt to the heterogeneity of CTCs in immunological approaches. More recently, a microfluidic-based device, called the “CTC-Chip”, has been developed for CTC cell detection strategies with a significant increase in yield and purity. Using this new technology, in which whole blood is flowing through chips with automatically optimized flow kinetics, microposts coated with anti-EpCAM antibodies capture CTCs directly from small volumes of blood samples. The CTCs are then stained with secondary antibodies against either CKs or tissue-specific markers, such as PSA in prostate cancer or HER2 in breast cancer, followed by automated scanning of the microposts. Several studies have demonstrated the potent usefulness of this method due to its enhanced sensitivity and specificity, in that the higher number of isolated CTCs facilitates dynamic monitoring during a time course of cancer therapies. Moreover, recent technological progress has allowed for the isolation and analysis of single intact CTCs. These remarkable approaches should have major impacts and further under-

![Flow chart of current and potential applications of circulating tumor cell and circulating cell-free nucleic acids technologies.](image)

Figure 1  Flow chart of current and potential applications of circulating tumor cell and circulating cell-free nucleic acids technologies. Circulating tumor cells (CTCs): The blood samples from cancer patients are processed through various isolation/enrichment and detection techniques. A new in vivo approach allows the enrichment of CTCs directly from a peripheral vein of patients, using a wire functionalized by attachment of epithelial cell adhesion antibodies. CTCs are usually captured along with contaminating leukocytes. Various detection methods are utilized to detect the rare cell population in the bloodstream. Circulating cell-free nucleic acids (cfNAs): Plasma/serum are generally isolated with centrifuge techniques and subsequently processed for the extraction of specific nucleic acids. Cancer-specific alterations are most commonly analyzed in circulating DNA. Circulating mRNA has attracted increasing attention because of its stability in plasma/serum. More recently, long non-coding RNA in plasma was also evaluated as a potent biomarker in gastric cancer patients. CGH: Comparative genome hybridization; FACS: Fluorescence activated cell sorter; FISH: Fluorescence in situ hybridization; FAST: Fiberoptic array scanning technology; EPISPOT: Epithelial immunospot; qRT-PCR: Quantitative real-time polymerase chain reaction; LOH: Loss of heterozygosity.
standing of the biology and significance of those heterogeneous populations.

Although most of these technologies have used blood samples in vitro, a new revolutionary in vivo approach allows the enrichment of CTCs directly from a peripheral vein of patients (Figure 1). In this system, a structured medical Seldinger guidewire is functionalized with the attachment of EpCAM antibodies. The device is inserted into a peripheral vein, which enables the capture of a large number of CTCs from up to 1.5 L of blood over the duration of 30 min. Despite its potent utility, a large-scale study is required to verify its relevance and to eliminate the possibility of adverse effects.

**Techniques for the detection and identification of CTCs**

After enrichment of CTCs, identification procedures are conducted to investigate their genetic and biological profiles in detail. Various methodologies for this process have been advocated and developed in the past few decades, ranging from cytometric/protein-based approaches to polymerase chain reaction (PCR)-based approaches. The former approaches involve conventional methods, such as immunostaining for specific markers, fluorescence in situ hybridization (FISH) and comparative genomic hybridization, and newly developed methods, such as fiberoptic array scanning technology with high throughput in CTC screening and epithelial immunospot, which can detect proteins secreted from CTCs.

PCR-based detection of CTCs has evolved remarkably, especially after the introduction of the quantitative RT-PCR (qRT-PCR) technique, which can minimize possible false-positive results by using a certain “cutoff value” during the analysis process. Identification of appropriate DNA/RNA-based markers expressed by CTCs is considered critical in order to enhance the specificity and reliability of its detection. Therefore, conventional markers for CTCs, such as CKs and CEA, and other diverse markers have been investigated towards their possible clinical application in several malignancies. CTC-related markers and the introduction of profile analysis including microRNAs (miRNAs) features also might be useful to resolve these issues.

**CTC detection in patients with GC and its clinical relevance**

To date, many researchers have tried to detect CTCs in patients with GC and demonstrated its relevance to biological and oncogenic functions using various approaches. Table 1 represents a summary of previous reports, especially focusing on methodologies, targeted molecules and detection rates. Since its introduction, RT-PCR technology has become the most widely used approach to achieve a satisfactory detection rate despite the extremely low concentration of CTCs in the bloodstream. However, a high sensitivity of RT-PCR may cause an increase in false positive detection even in healthy controls. Therefore, some researchers have utilized multiple detection markers in an mRNA-based assay and suggested its potent usefulness. Of particular note, Wu et al have developed a sensitive assay using a high-throughput colorimetric membrane array, in which multiple markers, such as human telomerase reverse transcriptase (TERT), cytokeratin 19 (CK19), CEA and MUC1, are measured simultaneously and the combination of four markers serves as a prognostic indicator for overall survival and postoperative recurrence/metastasis in GC. Recently, non-coding RNAs, such as miRNAs and Piwi-interacting RNAs (piRNAs), have been proven to alter their expression in carcinogenesis and tumor progression, so these cancer-specific alterations have been reported to be useful for the detection of CTCs in GC. However, some of those reports, in which a mononuclear cell layer was used to isolate total RNA, may not reflect miRNAs originating only from CTCs because the possibility of contamination by leukocyte-originated RNAs cannot be excluded. The presence of miRNAs originating from peripheral blood cells has been demonstrated in the blood of both cancer patients and normal individuals, and furthermore, contamination from those miRNAs has been observed even for circulating cell-free miRNA analysis. Those issues should be addressed before proceeding to clinical practice, and moreover, exhaustive exploration to identify more sensitive miRNA/piRNA-related markers might be desirable to achieve an accurate assay.

Recurrence and metastasis are the most critical factors not only for predicting clinical outcome but also for the quality of life in patients with GC. As summarized in Table 2, accumulating reports have suggested the significance of CTC detection as a prognostic indicator by various approaches, including both the CellSearch System and RT-PCR/qRT-PCR methods. Hiraia et al examined CTCs in 130 gastrointestinal cancer patients involving 44 GC patients using the CellSearch System. Their results demonstrated that the metastatic GC patients with ≥ 2 CTCs (n = 15) had a significantly shorter overall survival rate than the metastatic GC patients with < 2 CTCs (n = 12) (P = 0.039). In a prospective study, Matsusaka et al also evaluated the relevance of CTCs to chemotherapy and clinical outcome using the CellSearch System. Their results showed that GC patients with ≥ 4 CTCs at 2 and 4 wk after the initiation of chemotherapy had significantly shorter overall survival and progression-free survival in comparison with GC patients with < 4 CTCs, whereas CTC status at baseline (i.e., before the initiation of chemotherapy) had no statistical association with clinical outcomes. These findings may imply the close relationship of CTC status and treatment response.

The majority of the studies using RT-PCR/qRT-PCR methods, which are also widely used for prognosis analysis, have relatively small numbers of cases. Under such circumstances, Mimori et al focused on one candidate marker, membrane type 1 matrix metalloproteinase (MT1-MMP) mRNA levels, based on results from cDNA microarray analysis, and consequently validated its relevance in a subsequent qRT-PCR based study involving more than 800 GC patients. As a result, MT1-MMP
| Characteristic and number of patients | Control (n) | Detection method | Detection rate/statistic value | Ref. |
|--------------------------------------|-------------|------------------|-------------------------------|------|
| Pre or post treatment                |             |                  |                               |      |
| 1-IV                                 | 9 (PB)      | RT-PCR           | CEA mRNA                      | 22.2%| Funaki et al[133] |
| 1-IV                                 | 20 (PB)     | RT-PCR           | CEA mRNA                      | 0%   | (Pt.) |
| 1-IV                                 | 49 (PB)     | RT-PCR           | CK19 mRNA                     | 0%   | (Ctrl.) |
| 1-IV                                 | 30 (PB)     | RT-PCR           | CK20 mRNA                     | 0%   | (Pt.) |
| Inoperable/metastatic                | 34 (PB)     | RT-PCR           | CEA mRNA                      | 0%   | (Ctrl.) |
| I, II, III, IV                       | 35 (PB)     | RT-PCR           | CK19 mRNA                     | 0%   | (Pt.) |
| I-IV                                 | 52 (PB)     | RT-PCR           | CK19 mRNA                     | 9.6% | (Pt.) |
|                                      |             |                  | CK20 mRNA                     | 9.6% | (Pt.) |
|                                      |             |                  |                               | 1%   | (Pt.) |
|                                      |             |                  |                               |       |       |
|                                      |             |                  |                               |       |       |
| I-IV                                 | 41 (PB)     | RT-PCR           | CEA mRNA                      | 22.2%| Nishida et al[64] |
| (36 with curative surgery)           |             |                  |                               | 33.3%| (during curative surgery) |
| (5 with inoperable)                  |             |                  |                               | 80%  | (inoperable Pt.) |
| I-IV                                 | 57 (PA)     | RT-PCR           | CEA mRNA                      | 17.5%| Miyazono et al[68] |
| 49 (PV)                              |             |                  |                               | 18.4%| PA: 17.5% |
| 51 (SVC)                             |             |                  |                               | 21.6%| PV: 18.4% |
|                                      |             |                  |                               |       | SVC: 21.6% |
| EGC, III (paired, after surgery during surgery and follow-up) | 29 (PB) | RT-PCR | CEA mRNA | 22.2% | (before curative surgery) |
|                                      |             |                  |                               |       | (after surgery) |
|                                      |             |                  |                               |       | Total: 24.1% |
|                                      |             |                  |                               |       | III: 26.7% |
|                                      |             |                  |                               |       | IIIb: 34.4% |
|                                      |             |                  |                               |       | Total: 34.4% |
| I-IV                                 | 106 (PB)    | RT-PCR           | CEA mRNA                      | 40.6%| (Ctrl.) |
| (during surgery)                     |             |                  |                               |       |       |
| I-IV                                 | 41 (PB)     | RT-PCR           | CEA mRNA                      | 24.4%| Koike et al[64] |
| I-IV                                 | 46 (PB)     | qRT-PCR          | CK20 mRNA                     | 27.8%| Friederichs et al[64] |
| (18 EGJ cancer)                      |             |                  |                               |       | (Pt. with EG cancer) |
| (28 with GC)                         |             |                  |                               |       | (tumor-free Ctrl.) |
|                                      |             |                  |                               |       | (with benign GI disease) |
| I-IV                                 | 59 (PB)     | qRT-PCR          | CEA mRNA                      | 21.4%| Ikuguchi et al[64] |
|                                      |             |                  |                               | 0%   | (Pt. with GC) |
|                                      |             |                  |                               | 1%   | (tumor-free Ctrl.) |
|                                      |             |                  |                               |       | (before surgery) |
|                                      |             |                  |                               |       | (after surgery) |
| I-IV                                 | 70 (PB)     | RT-PCR           | CK20 mRNA                     | 36.6%| Illert et al[64] |
| (41 with curative resection)         |             |                  |                               |       | (Pt. with curative resection) |
| (29 with residual tumor)             |             |                  |                               |       | (Pt. with residual tumor) |
| I-III                                | 46 (PB)     | RT-PCR           | CEA mRNA                      | 52.2%| Seo et al[64] |
| (paired, before and after surgery)   |             |                  |                               | 19.6%| (after surgery) |
| I-IV                                 | 52 (PB)     | RT-PCR           | c-Met mRNA                    | 61.5%| Uem et al[64] |
|                                      |             |                  | MUC1 mRNA                     | 5.6% | (Pt.) |
|                                      |             |                  |                               | 71.2%| (Ctrl.) |
|                                      |             |                  |                               | 8.3% | (Pt.) |
| I-IV                                 | 42 (PB)     | RT-PCR           | hTERT mRNA                    | 61.9%| Wu et al[64] |
|                                      |             |                  | CK19 mRNA                     | 69.0%| (Pt.) |
|                                      |             |                  | CK20 mRNA                     | 3.3% | (Pt.) |
|                                      |             |                  |                               | 61.9%| (Pt.) |
| Tani et al. [147] | CK19 mRNA↑ | P = 0.0127 | (before surgery) | Tani et al. [147] | CK19 mRNA↑ | P = 0.0022 | (after surgery) |
|------------------|------------|-------------|------------------|------------------|------------|-------------|----------------|
| 41 (PB) | 64 | (paired, before and after chemotherapy) | No data for Ctrl. | 21.9% (before chemotherapy) | 0% (Ctrl.) | Wu et al. [44] | 81.3% (Pt.) |
| 1—IV | 101 (PB) | 64 | (paired, before and after chemotherapy) | No data for Ctrl. | 21.2% (after chemotherapy) | 26.8% (TDB sample) | Kolodziejczyk et al. [146] | 55.6% (metastatic GC) | Hiraiwa et al. [55] | 98.6% (metastatic GC) |
| 1—IV | 101 (PB) | 80 | (2 wk after chemotherapy) | No data for Ctrl. | 18.8% (metastatic GC) | 32.7% (baseline) | Matsuoka et al. [26] | 13.7% (metastatic GC) | Bertazzoli et al. [150] | 97.1% (metastatic GC) |
| 1—IV | 101 (PB) | 14 | (4 wk after chemotherapy) | No data for Ctrl. | 30% (metastatic GC) | 18.8% (baseline) | Matsuoka et al. [26] | 18.8% (metastatic GC) | Bertazzoli et al. [150] | 97.1% (metastatic GC) |
| 1—IV | 101 (PB) | 29 | (control samples were used the calibrator source) | AUC = 0.772 | (Pt.) 45.5% | 47.8% (baseline) | Matsuoka et al. [26] | 32.7% (preoperative) | Bertazzoli et al. [150] | 97.1% (preoperative) |
| 1—IV | 101 (PB) | 64 | (control samples were used the calibrator source) | AUC = 0.772 | (Pt.) 45.5% | 47.8% (baseline) | Matsuoka et al. [26] | 32.7% (preoperative) | Bertazzoli et al. [150] | 97.1% (preoperative) |
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| 1—IV | 101 (PB) | 64 | (control samples were used the calibrator source) | AUC = 0.772 | (Pt.) 45.5% | 47.8% (baseline) | Matsuoka et al. [26] | 32.7% (preoperative) | Bertazzoli et al. [150] | 97.1% (preoperative) |
mRNA levels in peripheral blood were indicated to be an independent factor for determining recurrence and distant metastasis of GC \((P = 0.0018)\).

Intriguingly, some groups have reported time-dependent changes in the detection rate of CTCs during the peri-operative time course\([58-61]\). Those changes may imply the possibility of monitoring the tumor dynamics; however, the biological and clinical meaning of CTCs still remains unknown and controversial. In fact, incontrollable events, including both increase and decrease in CTC detection rates during surgical maneuvers, have been proposed so far\([58-61]\). This discrepancy might be partially explained by a wide variety of measurement parameters, from the methodology itself to targeted markers, patient background/properties and sample conditions.

In summary, recent technological advances have provided considerable progress and interest in the detection of CTCs in various cancers, including GC. Although previous studies have shown a potent usefulness of CTC detection as a novel diagnostic and prognostic assay in cancer patients, little remains known about the biological features and fundamental roles of these cells. Detailed characterization of CTCs and well-designed experiments should resolve current underlying issues and provide the opportunity for clinical impact in cancer therapy.

### BIOLOGY AND DETECTION OF CELL-FREE NUCLEIC ACIDS

The study of cfNAs has a considerably long history since it was first reported in 1948 by Mandel and Metz\([62]\), who successfully detected nucleic acids in human plasma. Unfortunately, their work attracted little attention at that time owing to a lack of sufficient understanding of that innovative concept. Regarding malignant disease, in 1977, Leon et al\([63]\) first reported the presence of cell-free DNA (cfDNA) in the serum of cancer patients. Furthermore, they also mentioned its potent function as a clinical indicator, showing decreased cfDNA levels in response to radiotherapy. In 1989, Vasioukhin et al\([64]\) successfully detected cfDNA with neoplastic characteristics and proposed the first evidence suggesting that tumors can shed DNA into the circulation. This hypothesis was further strengthened by two studies in 1994, in which NRAS mutations in the plasma of patients with myelodysplastic syndrome or acute myelogenous leukemia, and KRAS mutations in the plasma or serum of patients with pancreatic cancer\([65]\), were detected. Those findings opened up a new field in the exploration of circulating nucleic acids, and many meritorious studies have demonstrated the biological function of cfNAs and their potential as novel biomarkers regarding DNA, mRNA and miRNAs (Figure 1).

In regard to the origin of circulating nucleic acids, two main potent release mechanisms, called “passive” and “active”, are advocated to date. The passive mechanism involves the release of nucleic acids originated from apoptotic and necrotic cells into the bloodstream. Macrophages and phagocytes play an important role in phagocytosis of necrotic and apoptotic cells and can release digested nucleic acids into the microenvironment\([66,67]\). In contrast, it is reported that fragments of cellular nucleic acids can be actively released\([68,69]\). Although the active secretion into the circulation remains enigmatic, one potential explanation is that cancer cells would release nucleic acids to transform the targeted recipient cells at distant locations\([70-72]\). In addition to those two mechanisms, cfNAs might be released by CTCs, however, there appears to be a huge gap between the amount of cfNAs and the rarity of CTCs in the bloodstream as described in the previous section. Thus, this hypothesis has been controversial so far.

#### Circulating cell-free DNA in plasma/serum

The study of circulating cfDNA in the plasma/serum involves the measurement of the total volume of circulating DNA as well as the detection of cancer-related genetic/epigenetic aberrations, which include microsatellite instability, loss of heterozygosity, genetic polymorphisms, point mutations, methylation, deletion/amplification/translocation of chromosome and integrity (i.e., the ratio of longer DNA fragment to shorter one based on the different cleavage process between apoptosis and necrosis\([73]\)). The latter approach is generally recognized to be able to cover a wider range of oncogenic alterations in various cancers and to possess more potent application in the clinical setting than the former one, partly because cfDNA can be released into the bloodstream and is

| CK20 mRNA | TFF1 mRNA | MUC2 mRNA | miR-21↑ | miR-200c↑ | miR-421↑ |
|-----------|-----------|-----------|---------|-----------|---------|
| 37.1%     | 31.4%     | 22.9%     | (Pt.)   | (Pt.)     | (Pt.)   |
| 100% (PB) | 0% (PB)   | 2% (PB)   | No detection in controls | AUC = 0.853 | AUC = 0.715 |
| 37.1%     | 31.4%     | 22.9%     | (Pt.)   | (Pt.)     | (Pt.)   |
| 100% (PB) | 0% (PB)   | 2% (PB)   | No detection in controls | AUC = 0.853 | AUC = 0.715 |
| 37.1%     | 31.4%     | 22.9%     | (Pt.)   | (Pt.)     | (Pt.)   |

AUC: Area under the receiver operating characteristic curve; BM: Bone marrow; EGC: Early gastric cancer; EGJ: Esophagogastric junction; GI: Gastrointestinal; MAH: Membrane-array hybridization; PA: Peripheral artery; PB: Peripheral blood; PVS: Portal vein; SVC: Superior vena cava; TDB: Tumor-draining blood; N/A: Not available; qRT-PCR: Quantitative real-time polymerase chain reaction; FACS: Fluorescence activated cell sorter; ICC: Intracellular cytokine flow cytometry.
### Table 2 Prognostic value of circulating tumor cells in gastric cancer

| Characteristic and number of patients | Detection method | Statistic value | Ref. |
|--------------------------------------|------------------|-----------------|------|
| 17 (non-responsive to chemotherapy) | RT-PCR CK19 mRNA | OS | $P = 0.014$ | Yeh et al. [55] |
| 57 1-IV | RT-PCR CEA mRNA | Liver metastasis recurrence | $P < 0.001$ | Miyazono et al. [9] |
| 106 1-IV | RT-PCR CEA mRNA | Recurrence/metastasis | $P = 0.02$ | Sumikawa et al. [64] |
| 46 1-IV (with curative resection) | qRT-PCR CK20 mRNA | 2-year-survival OS | $P = 0.03$ | Friederichs et al. [140] |
| 41 1-IV | RT-PCR CK20 mRNA | OS | $P = 0.0363$ | Illert et al. [144] |
| 46 1-III | RT-PCR CEA mRNA | Recurrence | $P < 0.00022$ | CEA after surgery (+) (a) |
| 52 1-IV | RT-PCR C-Met mRNA | OS | $P = 0.015$ | Seo et al. [45] |
| 42 1-IV | RT-PCR C-Met mRNA | OS | $P = 0.0178$ | Uen et al. [90] |
| 64 1-IV | MAH hTERT/CK19/CEA/MUC1 | Recurrence/metastasis | $P = 0.0018$ | Uen et al. [90] |
| 27 Metastatic | CellSearch System EpCAM EpCAM CK8/18/19 | OS | $P = 0.0223$ | Hiraiwa et al. [143] |
| 69 1-IV (with curative resection) | qRT-PCR CK19 mRNA | OS | $P < 0.001$ | Koga et al. [18] |
| 810 1-IV | RT-PCR MT1-MMP | OS | $P < 0.001$ | Mimori et al. [157] |
| 55 1-IV | RT-PCR ELISA Survivin mRNA | OS | $P < 0.001$ | Yie et al. [140] |
| 70 1-IV | qRT-PCR Survivin mRNA | OS | $P < 0.001$ | Bertaza et al. [134] |
| 51 Advanced | Cell search system EpCAM EpCAM CK8/18/19 | PFS (2 wk after chemotherapy) | $P = 0.001$ | Matsusaka et al. [96] |
| 48 4 wk after chemotherapy | OS | PFS (2 wk after chemotherapy) | $P < 0.001$ | (b) |
| 123 1-IV | qRT-PCR CEA mRNA | Recurrence DFS | $P = 0.001$ | Qiu et al. [52] |
| 30 1-IV (after curative surgery) | qRT-PCR CK18 mRNA | RFS | $P = 0.001$ | Saad et al. [151] |
| 95 1-IV | qRT-PCR B7-H3 mRNA | OS | $P = 0.02$ | Arigami et al. [156] |
| 98 1-IV | RT-PCR Survivin mRNA | DFS | $P = 0.001$ | Cao et al. [151] |
| 52 1-IV | qRT-PCR miR-200c | OS | $P = 0.016$ | Valladares-Ayerbe et al. [151] |

*a: $^2$ test/Fisher’s exact test; b: Kaplan-Meier survival curves, Log-rank test/Breslow-Wilcoxon test; c: Logistic regression model (multivariate); d: Multivariate Cox proportional hazard regression model. DFS: Disease-free survival; MAH: Membrane-array hybridization; OS: Overall survival; PFS: Progression-free survival; qRT-PCR: Quantitative real-time polymerase chain reaction; RFS: Relapse-free survival.*
detectable in the plasma/serum in healthy humans. In fact, numerous reports have demonstrated the detection of genetic and epigenetic alterations in circulating DNA in the plasma/serum in cancer patients. Furthermore, in colorectal cancer, recent two reports clearly demonstrated a correlation between acquired resistance to the anti-EGFR antibody drugs, such as cetuximab and panitumumab, and the emergence of KRAS mutations, which was successfully detected and monitored in the blood of patients under treatment. Misale et al. also indicated the potential of cfDNA to monitor tumor dynamics more sensitively compared to conventional assays, showing that KRAS mutant alleles were confirmed in the blood of a cetuximab-treated patient 10 mo earlier than radiographic examinations. Moreover, Leary et al. have recently analyzed individual tumor-specific DNA translocations in paired solid tumor and circulating cfDNA samples using next-generation sequencing technology and consequently demonstrated the feasibility of personalized biomarkers, enabling a so-called “tailor-made” therapeutic strategy. In summary, moving toward the development and future application in the clinical setting, the accumulated evidence has proven the potent usefulness of cfDNA for the detection of disease as well as for the assessment of residual disease, recurrence, and secondary resistance.

Detection of circulating DNA in patients with GC and its clinical relevance

Previous reports regarding circulating cfDNA in GC patients are summarized in Table 3. Among those reports, a few studies with respect to the concentration of circulating cfDNA are found, in which a housekeeping gene, beta-actin, and a non-coding genomic DNA repeat sequence, ALU, were evaluated. In contrast, the detection of methylated DNA in plasma/serum appears to be the most widely used approach in GC, which was usually investigated by methylation specific-PCR (MSP) or quantitative methylation specific-PCR (qMSP) assays. In 2002, Lee et al. first reported the potent application of detecting methylated DNA of death-associated protein kinase, E-cadherin, GSTP1, p15 and p16 in the serum of GC patients. Thereafter, technological advances and the exploration of more sensitive and specific genes have provided the accumulated evidence in this field. In detail, comprehensive analyses by methylation CpG island microarray have suggested the possibility of more significant genes for detecting methylated DNA. Most recently, Ling et al. clearly demonstrated the potential usefulness of detecting methylated XAF1 DNA as a diagnostic as well as prognostic biomarker with satisfactory degrees of specificity and sensitivity. Specifically, methylated XAF1 DNA in serum was detected in 69.8% (141/202) of the GC patients and none of the healthy individuals (0/88) with an area under the receiver operating characteristic curve (AUC) of 0.909 in a receiver operating characteristic (ROC) curve analysis for discrimination of the two groups and was significantly correlated with poorer prognosis in GC (P < 0.001, disease-free survival, Kaplan-Meier survival curves, Log-rank test).

Concerning genetic alterations in other types of cancers, the relationships with tumor-specific gene alteration such as HER2 in breast cancer and adenosomatous polyposis coli in colorectal cancer have been revealed even in circulating cfDNA. In GC, Park et al. investigated gene amplification of MYC in the plasma of GC patients and showed that the plasma MYC/GAPDH ratio was significantly higher in the GC patients than that in the healthy controls (P < 0.001) and correlated with the tissue MYC/GAPDH ratio (P = 0.009), and tissue MYC status by FISH (P = 0.024). In contrast, among GC patients with a 2+ or 3+ score in a HER2 IHC assay, Lee et al. reported that no significant association was observed between the HER2 level in plasma and the copy number variation in tumor tissue determined by FISH. Although it is unclear why there was a discrepancy between these two results, it may be partially explained by the inappropriate employment of reference genes and the heterogeneity of GC tissues. The investigation of circulating cfDNA relating to genetic aberration in GC remains in its infancy. Therefore, further evidence is expected to address current controversial issues and develop this field.

Circulating cell-free mRNA in plasma/serum

The presence of RNase in plasma/serum had long been known, and furthermore, the RNase concentration in serum was reported to be elevated in cancer patients in the 1970s. Given that mRNA in plasma/serum might be more fragile than DNA and susceptible to degradation by RNase, it was not clear whether mRNA could exist in plasma/serum with sufficient integrity to allow amplification, although several reports had previously suggested the possible presence of RNA in serum forming a complex with protelipids. In 1999, two groups reported the successful detection of cell-free RNA such as tyrosinase mRNA in serum of patients with malignant melanoma and Epstein-Barr virus-associated RNA associated in plasma of patients with nasopharyngeal carcinoma. Subsequently, many studies have demonstrated the presence of specific mRNA in plasma/serum and its potent clinical relevance in patients with a variety of cancers. At present, it is considered that mRNA in plasma/serum may be protected from degradation by packaging in secretory membrane vesicles, such as exosomes, microvesicles and multivesicles, which are released from cellular surfaces into the bloodstream.

Circulating cell-free miRNA in plasma/serum

In the past decade, circulating cell-free miRNAs in plasma/serum have attracted increasing attention among investigators in various types of research field including oncology. The discovery of miRNA dates back to 1993, when Lee et al. found that a short RNA product encoded by the lin-4 gene inhibited the translation of its putative target, lin-14 mRNA, with partial sequence complementarity during a study of Caenorhabditis elegans (C.
Table 3  Circulating cell-free DNA in gastric cancer

| Characteristic and number of patients | Controls (n) | Plasma/serum Detection method | Detection rate/statistic value | Ref. |
|--------------------------------------|-------------|-------------------------------|-------------------------------|------|
| Unresectable                         | 198         | Serum/MSP                      | 82.8% (GC)                   | Ren et al[152] |
|                                      | 78 (peptic ulcer) | Immuno-PCR                     | 7.7% (peptic ulcer)             |     |
|                                      | 118 (chronic gastritis) | Semi quantitative PCR          | 5.9% (chronic gastritis)       |     |
|                                      | 236 (healthy donors) |                          | 0.8% (healthy donors)          |     |
|                                      |             |                               | \( P < 0.01 \)                 |     |
| N/A                                  | 51          | Serum/mSP                      | 100%                          | Lo et al[159] |
|                                      | 30 (gastritis) | qPCR                           | 92.9%                         |     |
|                                      | 197 (healthy controls) | EBV DNA          | 26.1%                         | Kanyama et al[89] |
|                                      |             |                               | No detection in controls      |     |
| I-Ⅳ                                  | 54          | Serum/MSP                      | 48.1%                         | Lee et al[80] |
|                                      | 30          | DAP-kinase                     | 36.7%                         |     |
|                                      |             | E-cadherin                    | 18.3%                         |     |
|                                      |             | GSTP1                         | 23.9%                         |     |
|                                      |             | p15                            | 51.9%                         |     |
|                                      |             | p16                            | 55.6%                         |     |
|                                      |             | p16 + E-cadherin + RARb       | 57.4%                         |     |
|                                      |             | E-cadherin                    | 14.8%                         |     |
|                                      |             | p16 + E-cadherin + RARb + E-cadherin | 41.6% |     |
| I-Ⅳ                                  | 60          | Serum/MSP                      | 27.0%                         | Kanyama et al[89] |
|                                      | 16          | p16                            | 23.8%                         |     |
|                                      |             | No detection in controls      | 17.5%                         |     |
|                                      |             | E-cadherin                    | 24.4%                         |     |
|                                      |             | RARb                           | 22.0%                         |     |
|                                      |             | p16 + E-cadherin + RARb + E-cadherin | 22.0% |     |
| I-Ⅳ                                  | 109         | Serum/MSP                      | 22.0%                         | Ichikawa et al[84] |
|                                      | 10          | p16                            | 27.0%                         |     |
|                                      |             | E-cadherin                    | 18.3%                         |     |
|                                      |             | p16 + E-cadherin + RARb       | 23.9%                         |     |
|                                      |             | E-cadherin                    | 36.7%                         |     |
|                                      |             | No detection in controls      | 51.9%                         |     |
| I-Ⅳ                                  | 63          | Serum/MSP                      | 23.3%                         | Koike et al[42] |
|                                      | 10          | p16                            | No detection in controls      |     |
|                                      |             | E-cadherin                    | 22.0%                         |     |
|                                      |             | RARb                           | 22.0%                         |     |
|                                      |             | p16 + E-cadherin + RARb + E-cadherin | 14.6% |     |
| I-Ⅳ                                  | 60          | Serum/mSP                      | No detection in controls      | Leung et al[42] |
|                                      | 22          | qMSP                           | Four markers combined         |     |
|                                      |             | APC                            | 16.7%                         |     |
|                                      |             | E-cadherin                    | 13.3%                         |     |
|                                      |             | hMLH1                          | 41.7%                         |     |
|                                      |             | TIMP3                          | 16.7%                         |     |
|                                      |             | No detection in controls      | 55%                           |     |
|                                      |             | RARb                           | 13.6%                         |     |
|                                      |             | p16 + E-cadherin + RARb + E-cadherin | 51.6% |     |
|                                      |             | APC + E-cadherin              | 13.6%                         |     |
|                                      |             | p16 + E-cadherin + RARb       | 47.7%                         |     |
|                                      |             | No detection in controls      | \( P = 0.006 \)               |     |
|                                      |             | Methylation (+) vs (-)        | (b)                           |     |
| I-Ⅳ                                  | 109         | Serum/MSP                      | 23.8%                         | Ikoma et al[204] |
|                                      | 10          | RARb                           | 47.7%                         |     |
|                                      |             | p16 + E-cadherin + RARb + E-cadherin | 41.7% |     |
| I-Ⅳ                                  | 53          | Plasma/mSP                     | 23.8%                         | Sai et al[84] |
|                                      | 21          | qPCR                           | \( P = 0.03 \)                 |     |
|                                      |             | β-actin (102 bp)              | \( P < 0.0001 \)              |     |
|                                      |             | No detection in controls      | AUC = 0.75                    |     |
|                                      |             | DNA integrity (253 bp/102 bp)  | \( P = 0.07 \)                 |     |
| N/A                                  | 4           | Serum/MSP                      | 100%                          | Tan et al[414] |
|                                      | 10          | RUNX3                          | 50%                           |     |
|                                      |             | p16                            | 25%                           |     |
|                                      |             | RASSF1A                        | 25%                           |     |
|                                      |             | CDH1                           | 25%                           |     |
| I/IV | No. | No. | Type | Method | Gene | Control | Tani et al. [147] | Tsujiura M et al. Liquid biopsy of gastric cancer patients |
|------|-----|-----|------|--------|------|---------|----------------|--------------------------------------------------|
| I/IV | 52  | 20  | Serum | MSP   | p16  | 9.6%    | 0%             | Abbassazadegan et al. [165]                       |
|      |     |     |       |       | E-cadherin | 9.6% | 60.9% (Pt. with p16 methylation in primary tumor) |
| I/IV | 52  | 50  | Serum | MSP   | p16 + E-cadherin + RARb | 23.1% | (all Pt.) |
|      |     |     |       |       | p16 | 26.9% | (Ctrl.) |
| I/IV | 20  | 22  | Plasma| Fluorescence-based assay | MSP | DNA concentration↑ | Pt. (n = 20) vs Ctrl. (n = 22) ( Validation 1) |
|      |     |     |       | DNA concentration↑ | MGMT | 70% | Pt. (Ctrl.) |
|      |     |     |       |       | p15 | 36.4% | (Pt.) |
|      |     |     |       |       | hMLH1 | 50% | (Ctrl.) |
|      |     |     |       |       |     | 18.2% | (Pt.) |
|      |     |     |       |       |     | 25% | (Ctrl.) |
|      |     |     |       |       |     | 9.1% | (Pt.) |
|      |     |     |       |       |     | 24.0% | (Pt.) |
| I/IV | 47  | 30  | Serum | MSP | RASSF1A | 23.1% | 10% (benign gastric disease) |
|      |     |     |       |       |     | 3.3% | (healthy controls) |
| I/IV | 57  | 79  | Plasma| qPCR | MYC/ GAPDH↑ | 36.4% | 55% (Pt.) |
|      |     |     |       |       |     | 19.0% | (Ctrl.) |
|      |     |     |       |       |     | 0% | Pt. (n = 57) vs Ctrl. (n = 29) ( Validation 2) |
| I/IV | 65  | 50  | Serum | qMSP | RUNX3 | 29.2% | 29.2% (Pt.) |
|      |     |     |       |       |     | 0% | (Ctrl.) |
|      |     |     |       |       |     | 10% | Pt. (n = 65) vs Ctrl. (n = 50) (Validation 1) |
| I/IV | 65  | 40  | Serum | MSP | DLEC1 | 33.8% | 3.3% (benign gastric disease) |
|      |     |     |       |       |     | 5% | (healthy controls) |
| I/IV | 73  | 20  | Serum | qMSP | TFPI2 | 9.6% | 0% (healthy controls) |
|      |     |     |       |       |     | 0% | (Ctrl.) |
|      |     |     |       |       |     | 5% | Correlation with LN meta. (a) |
|      |     |     |       |       |     | P = 0.004 | Correlation with distant meta. (a) |
| I/IV | 65  | 80  | Plasma| qMSP | SLC19A3 | 29.2% | 0% (validation 1) |
|      |     |     |       |       |     | 5% | Pt. (n = 45) vs Ctrl. (n = 60) (Validation 2) |
| I/IV | 46  | 30  | Serum | Methylation CpG island microarray | MSP | 56.5% | AUC = 0.841 (Validation 1) |
|      |     |     |       |       |       | 3.3% | (Ctrl.) |
|      |     |     |       |       |       | 10% | Pt. (n = 20) vs Ctrl. (n = 20) (Validation 2) |
| I/IV | 58  | 30  | Serum | MeDIP | FAM5C | 31.0% | 70.7% Pre- vs post-operation |
|      |     |     |       |       |       | 70.7% | P < 0.001 Pre- vs post-operation (GC Pt.) |
|      |     |     |       |       |       | 77.6% | (Healthy controls) |
|      |     |     |       |       |       | 10% (gastric precancerous lesions) |
### Detection of circulating cell-free RNA in patients with GC and its clinical relevance

As summarized in Table 4, the number of previous reports regarding circulating mRNA in GC patients is small compared with those regarding other types of cancers and with those concerning circulating DNA in GC. However, as Kang et al. recently reported that the detection of plasma hTERT mRNA can serve as a potential marker for diagnosis and prognosis of GC patients, increased insight and evidence about circulating mRNA might facilitate the development of this field.

Since the potent utility of determining miRNAs in the plasma of GC patients was first reported by our group in 2011[12], many studies have demonstrated the significance of circulating miRNAs as novel biomarkers (Table 5). Still, the immaturity of the field has led to several issues concerning its actual introduction in clinical settings. To date, there has been no consensus regarding how inter- and intra-individual variations can affect the results, which sample (i.e., plasma or serum) is more favorable for diagnosis and prognosis of GC patients, increased insight and evidence about circulating mRNA might facilitate the development of this field.

### Methylation specific-PCR (qMSP).

#### Table 1: miRNA expression in GC patients

| LN | N/A | Pt. with 2+/3+ score in HER2 IHC assay | Pt. | Ctrl. | Pt. | Ctrl. | Pt. | Ctrl. |
|----|-----|---------------------------------------|-----|-------|-----|-------|-----|-------|
| Serum | ATP4B | AUC = 0.784 | 64% | 0% | 64% | 0% | 64% | 0% |
| Serum | Vimentin | P = 0.018 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |
| Serum | SOX17 | OS: P = 0.049 | 64.4% | 0% | 64.4% | 0% | 64.4% | 0% |
| Serum | XAF1 | AUC = 0.909 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |

#### Table 2: miRNA expression in GC patients

| LN | N/A | Pt. with 2+/3+ score in HER2 IHC assay | Pt. | Ctrl. | Pt. | Ctrl. | Pt. | Ctrl. |
|----|-----|---------------------------------------|-----|-------|-----|-------|-----|-------|
| Serum | ATP4B | AUC = 0.784 | 64% | 0% | 64% | 0% | 64% | 0% |
| Serum | Vimentin | P = 0.018 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |
| Serum | SOX17 | OS: P = 0.049 | 64.4% | 0% | 64.4% | 0% | 64.4% | 0% |
| Serum | XAF1 | AUC = 0.909 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |

#### Table 3: miRNA expression in GC patients

| LN | N/A | Pt. with 2+/3+ score in HER2 IHC assay | Pt. | Ctrl. | Pt. | Ctrl. | Pt. | Ctrl. |
|----|-----|---------------------------------------|-----|-------|-----|-------|-----|-------|
| Serum | ATP4B | AUC = 0.784 | 64% | 0% | 64% | 0% | 64% | 0% |
| Serum | Vimentin | P = 0.018 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |
| Serum | SOX17 | OS: P = 0.049 | 64.4% | 0% | 64.4% | 0% | 64.4% | 0% |
| Serum | XAF1 | AUC = 0.909 | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) | (Pt.) | (Ctrl.) |
increasing owing to recent advances in bioinformatic analysis, making it more difficult to obtain a meticulous understanding of each miRNA. Although comprehensive approaches by genome-wide profiling can address those problems to some extent, a further large-scale validation with well-established methods seems to be required in this area as well.

To overcome obstacles due to inter-individual variations, our group tried to identify candidate miRNAs by comparing miRNA profiles between pre- and post-operative samples in the same individuals. Because miRNAs are involved in various non-cancerous cell biology including physiological modulation and pathological disruption of basic pathways, the existence of inter-individual differences can be strongly suspected based on miRNA expression. As a result, two miRNAs, miR-451 and miR-486, were selected based on this strategy and their significant value in discriminating between GC patients and healthy controls was clearly demonstrated with an AUC of 0.96 and 0.92 in ROC curve analysis for miR-451 and miR-486, respectively. We suggest that the miRNAs isolated by these concepts could be valuable biomarkers for the effective detection of early cancer and recurrence because the change of these miRNAs can be affected by the reduction of the volume of cancer tissue and is therefore directly related to tumor existence.

Most recently, our group published new observations in which long non-coding RNAs (lncRNAs) in the plasma of GC patients were successfully detected. Specifically, three lncRNAs (H19, HOX antisense intergenic RNA and metastasis associated lung adenocarcinoma transcript-1) stably exist in plasma from both GC patients and healthy controls. Plasma H19 levels were significantly higher in the patient group than the control group and decreased postoperatively, implying the possible use of H19 levels as a novel diagnostic marker in GC. LncRNAs are defined as non-protein coding transcripts longer than 200 nt lacking significant open reading frames and involved in fundamental cellular processes, such as RNA processing, gene regulation, chromatin modification, gene transcription, and post-transcriptional gene regulation based on RNA sequence complementary interactions. Detailed investigations have shown that lncRNAs can exhibit developmental and tissue specific expression patterns as well as aberrant regulation in a variety of diseases, including GC. Explorations of a novel type of RNA can provide more intriguing aspects in this research field.

**CONCLUSION**

Although the concept of “liquid biopsy” possesses great potential in detection and monitoring of diseases as previously described in detail, several hurdles should be
**Table 5  Circulating cell-free microRNA/long non-coding RNA in gastric cancer**

| Characteristic and number of patients | Controls (n) | Plasma/serum | Detection method | Detection rate/statistic value | Ref. |
|--------------------------------------|-------------|--------------|-----------------|--------------------------------|------|
| miR-145†                            | 69          | Plasma       | qRT-PCR         | P = 0.01                        |      |
| miR-21†                             | 60          | Plasma       | qRT-PCR         | P = 0.016                       | Tsujiura et al[125] |
| miR-21‡                             | 60          | Plasma       | qRT-PCR         | P = 0.01                        | Konishi et al[126] |
| miR-187††                           | 40          | Serum        | qRT-PCR         | P = 0.0016                      | Liu et al[127] |
| miR-371-5p†                         | 40          | Serum        | qRT-PCR         | P = 0.009                       | Liu et al[128] |
| miR-378†                            | 82          | Serum        | Microarray      | AUC = 0.74                       | Song et al[129] |
| miR-376c†                           | 82          | Serum        | qRT-PCR         | AUC = 0.71                       |      |
| miR-444†                            | 82          | Serum        | qRT-PCR         | AUC = 0.71                       |      |
| miR-196a                            | 20          | Plasma       | qRT-PCR         | P = 0.012                       | Tsai et al[130] |
| miR-20a‡                            | 30          | Plasma       | qRT-PCR         | P = 0.003                       |      |
| miR-21†                            | 39          | Serum        | qRT-PCR         | P = 0.001                       | Wang et al[131] |
| miR-21†                            | 39          | Serum        | qRT-PCR         | P = 0.001                       | Wang et al[132] |
| miR-375†                            | 20          | Serum        | miRNA microarray | P = 0.001                       | Zhang et al[133] |
| miR-195-5p†                         | 20          | Plasma       | qRT-PCR         | P = 0.059                       | Gorur et al[134] |
| miR-211†                            | 79          | Plasma       | miRNA microarray | P = 0.001                       | Kim et al[135] |
| miR-146a†                           | 79          | Plasma       | miRNA microarray | P = 0.001                       | Kim et al[135] |
| miR-148a†                           | 79          | Plasma       | miRNA microarray | P = 0.001                       | Kim et al[135] |
| CISH: P = 0.0451                     |             |              |                 |                                |      |

**Note:** Detection rate/statistic value is based on the comparison of microRNA expression levels between different groups. P-values indicate the significance of the differences.
overcome before translating it into clinical settings. One of the most important issues is the lack of consensus in technical approaches, which involves various aspects of the methodologies, such as preferable sample type, storage conditions, candidate molecules and suitable detection techniques. Moreover, technical errors may introduce contaminated cells or molecules into experimental samples, which could cause misunderstandings and statistical errors. Therefore, the standardization of techniques throughout all experimental steps should be emphasized.

Owing to recent remarkable technological developments, novel revolutionary approaches including an in vitro CTC isolation system[^32] and multi-detectable array have been introduced into this research field. However, some issues raised by those advances should be addressed properly. Although multi-detection approaches can facilitate exhaustive screenings and provide us with various types of information, the important considerations are which molecules should be selected as a tumor marker and how the result of an individual patient obtained by multiple detection panels should be effectively utilized. Of course, the cost and practicality of each assay should also be taken into consideration to some extent.

In summary, the science of CTCs and circulating cfNAs remains in its infancy. Despite numerous approaches and techniques that have been advocated to accomplish the ultimate goal, that is, the development of a useful, sensitive and real-time monitoring system from the blood, few proposals have been translated into clinical practice. Large-scale studies and further understanding of their biology and significance could resolve those problems and enhance their utility as biomarkers. Consequently, the development of novel biomarkers based on CTCs and cfNAs could provide many benefits for cancer patients including the improvement of clinical outcomes in the near future.

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