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Clinica Chimica Acta 363 (2006) 187 – 196

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Review

Diagnostic developments involving cell-free (circulating) nucleic acids☆

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Received 3 April 2005; received in revised form 6 May 2005; accepted 16 May 2005
Available online 26 August 2005

Abstract

Background: The detection of circulating nucleic acids has long been explored for the non-invasive diagnosis of a variety of clinical conditions. In earlier studies, detection of circulating DNA has been investigated for the detection of various forms of cancer. Metastasis and recurrence in certain cancer types have been associated with the presence of high levels of tumor-derived DNA in the circulation. In the case of pregnancies, detection of fetal DNA in maternal plasma is a useful tool for detecting and monitoring certain fetal diseases and pregnancy-associated complications. Similarly, levels of circulating DNA have been reported to be elevated in acute medical emergencies, including trauma and stroke, and have been explored as indicators of clinical severity. Apart from circulating DNA, much attention and effort have been put into the study of circulating RNA over the last few years. This area started from the detection of tumor-derived RNA in the plasma of cancer patients. Soon after that, detection of circulating fetal RNA in maternal plasma was described. Plasma RNA detection appears to be a promising approach for the development of gender- and polymorphism-independent fetal markers for prenatal diagnosis and monitoring. This development also opens up the possibility of non-invasive prenatal gene expression profiling by maternal blood analysis. Besides circulating DNA and RNA in plasma and serum, cell-free DNA in other body fluids, such as urine, has been detected in patients with different clinical conditions. Regardless of the sources of cell-free DNA for clinical use, the amount is frequently scarce.

Methods: Technical advancements in detecting free DNA have been made over the years.

Conclusions: It is likely that further developments in the field of circulating nucleic acids will provide us with new diagnostic and monitoring possibilities over the next few years.

Keywords: Circulating nucleic acids; Plasma/serum; Cancer; Prenatal diagnosis; Rapid testing

Contents

☆ YMDL and YKT are supported by the Innovation and Technology fund (ITS/195/01).

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doi:10.1016/j.cccn.2005.05.048

1. History of circulating nucleic acids

The discovery of cell-free nucleic acids in the circulation was first reported in 1948 by Mandel and Metais, who were able to detect nucleic acids from human plasma [1]. This finding was remarkable but not widely recognized initially. For a long period of time, studies on circulating DNA were mainly focused on autoimmune diseases, such as rheumatoid arthritis and systemic lupus erythematosus, in which high levels of circulating DNA were detected in the serum of patients [2,3].

The potential application of circulating nucleic acids in the diagnosis and prognosis of cancer was first demonstrated in 1977, when high levels of circulating DNA were detected in the serum of cancer patients, and the levels of circulating DNA decreased when the patients responded to radiotherapy [4]. In 1989, Stroun et al. detected circulating DNA with neoplastic characteristics in the plasma of cancer patients [5]. A few years later, tumor-derived oncogene mutations were detected in the plasma or serum of patients with pancreatic cancer [6], myelodysplastic syndrome or acute myelogenous leukemia [7]. These findings confirmed that tumor-derived DNA could be isolated from the plasma or serum of cancer patients.

Besides cancer detection and monitoring, studies of circulating nucleic acids have opened up a new avenue for non-invasive prenatal diagnosis. This was made possible by the successful detection of fetal-derived Y-chromosomal sequences in maternal plasma and serum by Lo et al. in 1997 [8]. In addition to these applications, circulating nucleic acids have been demonstrated to be potentially useful in monitoring trauma [9,10] and stroke patients [11].

2. Application of circulating DNA

2.1. Cancer testing

Cancer development involves an accumulation of genetic and epigenetic changes, such as point mutations, chromosomal rearrangements, microsatellite instability, and promoter hypermethylation. After the first demonstration of detectable tumor-associated DNA sequences in the plasma of cancer patients by Stroun et al. in 1989 [5], different forms of tumor-derived circulating DNA were detected in patients with various types of cancers by different research groups.

In 1994, detection of N-ras gene mutations was reported in the plasma of patients with myelodysplastic syndrome or acute myelogenous leukemia [7], and that of K-ras gene mutations was reported in the plasma or serum of patients with pancreatic cancer [6]. Later, K-ras gene mutations were found in the circulating DNA and corresponding tumor tissues of pancreatic cancer patients [12–14]. In a report from Sorenson K-ras mutations were found in the plasma/serum of colorectal and pancreatic carcinoma patients [15]. K-ras mutations in the plasma or serum of colorectal cancer patients were also described by Kopreski et al. [16,17]. In some of these studies, persistence of mutated circulating K-ras sequences was related to recurrence or a progressive disease [14,18,19].

Chromosomal rearrangements represent another class of events that could occur in certain neoplasia. Frickhofen et al. demonstrated that rearranged Ig heavy chain DNA sequences are detectable in the plasma or serum from patients with non-Hodgkin's lymphoma or acute B-precursor lymphoblastic leukemia [20].

Microsatellite instability, in particular, loss of heterozygosity (LOH), is frequently found in solid tumors. Genetic changes corresponding to those found in tumor tissues were detected in the circulation of patients with various malignancies. Detection of microsatellite alterations in plasma DNA and serum DNA was first described in small cell lung cancer (SCLC) patients [21], and head and neck cancer patients [22], respectively. Subsequently, microsatellite alterations have been identified in the plasma or serum of patients suffering from clear cell renal carcinoma [23], colorectal cancer [24], non-small-cell lung cancer (NSCLC) [25], melanoma [26–28], ovarian cancer [29], breast cancer [30,31], lung cancer [32], acute myeloid leukemia and myelodysplasia [33]. In many of these studies, the levels and persistence of the microsatellite alterations were found to have prognostic correlations with disease progression or recurrence.

Promoter hypermethylation is another common molecular event that occurs in cancer development. Two groups simultaneously reported the detection of aberrantly methylated circulating DNA in cancer patients, using methylation-specific polymerase chain reaction (MSP) [34,35]. Other groups subsequently confirmed these studies and extended the spectrum of tumor types applicable to such a detection strategy [36–42]. The development of real-time MSP further allows a quantitative dimension in a number of such studies [43].

Detection of viral sequences was reported in the circulation of patients suffering from neoplasms associated with viral infections. Examples include the detection of Epstein–Barr virus (EBV) DNA in the serum of patients with nasopharyngeal carcinoma (NPC) [44], EBV-associated Hodgkin's disease [45,46], and post-transplant lymphoproliferative disease (PTLD) [47]. In addition, apart from tumor detection at presentation, such an approach also has implications for cancer monitoring and prognostication in patients with NPC [48–53] and EBV-associated lymphoid malignancies [54]. Besides EBV-associated malignancies, quantitative detection of human papillomavirus (HPV) DNA sequences in patients' sera was found to be a potential marker of early metastatic disease in HPV-associated head and neck squamous cell carcinoma (HNSCC) [55]. Moreover, detection of HPV DNA in plasma of cervical cancer patients was associated with disease metastasis [56].

2.2. Prenatal diagnosis

Conventional prenatal diagnostic procedures, such as chorionic villus sampling (CVS) or amniocentesis, impart a risk of fetal loss. The discovery of fetal DNA in maternal plasma and serum has opened up new opportunities in non-invasive prenatal diagnosis and monitoring [8]. By detecting Y-chromosome signals in maternal plasma with real time PCR, Honda et al. were able to achieve a 100% sensitivity from the fifth week of gestation [57]. Circulating fetal-associated DNA is of a higher abundance than fetal cells in the maternal circulation [58], and the post-partum clearance was shown to be rapid [59].

Fetal DNA in maternal plasma has been shown to be potentially useful for the prenatal diagnosis of certain neurological disorders [60], fetal chromosomal aneuploidies [61], sex-linked disorders [62], and fetal rhesus D (RhD) status [63]. Through the detection of paternally inherited genetic traits in maternal plasma, non-invasive exclusion of congenital adrenal hyperplasia was described by various groups [64,65]. In addition, Chiu et al. demonstrated an approach for the non-invasive exclusion of β-thalassemia by maternal plasma analysis [66]. Furthermore, fetal RhD genotyping from maternal plasma [67,68] has become an adopted protocol in routine prenatal diagnosis in several centers (e.g., see http://www.bloodnet.nbs.nhs.uk/ibgrl/Reference%20Services/RefSer_genotyping.htm).

Quantitative aberrations of fetal DNA in maternal plasma have been reported for various disease conditions, such as preterm labor [69,70], fetal chromosomal aneuploidies [71–73], preeclampsia [70,74–80], fetal–maternal hemorrhage [81], polyhydramnios [82], and invasive placentation [70,83]. However, these studies were based primarily on the detection of Y-chromosomal sequences in maternal plasma, an approach which has thus far limited their applications to the 50% of pregnancies involving male fetuses (Table 1).

Through the study of a single-nucleotide polymorphism (SNP) within a differentially methylated locus IGF2-H19, Poon et al. were able to detect fetal-derived maternally inherited alleles in maternal plasma [84]. This work represents the first use of epigenetic fetal markers for non-invasive prenatal diagnosis. Such an approach holds promise for the development of new gender- and polymorphism-independent fetal DNA markers for detection in maternal plasma.

2.3. Traumatology and stroke

Circulating DNA concentrations in plasma have been reported to be correlated with the severity of injury in trauma patients [9]. Another demonstration came from the correlation between plasma DNA levels and the severity of stroke [11]. In addition, high plasma DNA levels were reported to be associated with hospital mortality. With the development of rapid cycle PCR technology [85], such an approach may potentially be used in the emergency room.

3. Application of circulating RNA

3.1. Cancer testing

Using reverse transcriptase (RT) PCR, tumor-derived RNA was first reported to be detectable in plasma or serum of cancer patients with NPC [86] and melanoma [87]. Tumor-associated mRNA of various telomerase components was later shown to be detectable in serum or plasma of patients with breast cancer [88], colorectal cancer, follicular lymphoma [89] and hepatocellular carcinoma [90]. In addition, cytokeratin 19 (CK19) and mammaglobin mRNA was detectable in the plasma of breast cancer patients, and was associated with poor prognosis [91]. CK19 and carcinoembryonic antigen (CEA) RNA was detected in the plasma of colorectal cancer patients and was believed to be associated with advanced stages [92]. As another example, detection of beta-catenin mRNA in plasma of colorectal carcinoma and adenoma patients has recently been achieved and may serve as another potential marker for non-invasive cancer monitoring [93].

3.2. Prenatal diagnosis

Soon after the successful detection of tumor-derived RNA in plasma/serum of cancer patients, the presence of fetal RNA in maternal plasma was reported [94]. Further development in this area demonstrated that placental-derived mRNA species, such as human placental lactogen (hPL), the beta-subunit of human chorionic gonadotropin (βhCG), and corticotrophin-releasing hormone (CRH), are detectable in maternal plasma, and their expression is correlated with the corresponding protein product levels

Table 1
Quantitative assessments of fetal DNA in maternal plasma/serum for pregnancy-associated complications

Complication	Normal median[a] (number of cases)	Disease median[a] (number of cases)	Gestational age (weeks)	Body fluid	Reference (year)
Preterm labor	65.8 copies/ml (17)	124.8 copies/ml (13)	26–34	Plasma	[69] (1998)
	15.9 copies/ml (77)	44.6 copies/ml (35)	25–36	Plasma	[70] (2004)
Trisomy 21					
Boston samples	23.3 (19)	46.0 (7)	12–21	Plasma	[71] (1999)
Hong Kong samples	16.3 (18)	48.2 (6)	16–21	Plasma	[71] (1999)
	83.1 (29)	185.6 (15)	14+4	Plasma	[72] (2000)
Trisomy 18	83.1 (29)	95.9[b] (6)	14+4	Plasma	[72] (2000)
	40.3 (24)	31.5[b] (5)	15–20	Serum	[73] (2003)
Trisomy 13	83.1 (29)	213.2 (3)	14+4	Plasma	[72] (2000)
	40.3 (23)	97.5 (5)	15–20	Serum	[73] (2003)
Preeclampsia	76 (20)	381 (20)	27–41	Serum	[74] (1999)
	22.0 (33)	41.9 (18)	11–22	Plasma	[75] (2001)
	332.82 (46)	1599.07 (39)	28–42	Plasma	[76] (2001)
	227 (10)	521 (7)	30–38.3	Plasma	[77] (2002)
	128.5 copies/ml (40)	422.9 copies/ml (10)	19–25	Plasma	[78] (2002)
	191 (20)	486 (9)	29–36	Plasma	[79] (2003)
	22.4 copies/ml (50)	173.2 copies/ml (15)	29–38	Plasma	[70] (2004)
Han Chinese in Lhasa	90 (21)	810 (15)	35–41	Plasma	[80] (2004)
Tibetans in Lhasa	76.5 (27)	859.54 (11)	36–40	Plasma	[80] (2004)
Fetal growth restriction	191 (20)	141[b] (9)	29–36	Plasma	[79] (2003)
Fetal–maternal hemorrhage	Before ECV[c] 296 copies/ml (45)	After ECV[c] 369 copies/ml (45)	36+	Serum	[81] (2000)
Polyhydramnios	404 (14)	749.2 (1)	33+	Plasma	[82] (2000)
Invasive placenta (placenta previa)	184.2 (13)	294.3 (18)	31–36	Plasma	[83] (2002)
	37.0 copies/ml (69)	167.7 copies/ml (6)	27–36	Plasma	[70] (2004)

[a] Unless otherwise stated, median fetal DNA concentration is expressed as genome-equivalents/mL of maternal plasma or serum (Y-chromosome).
[b] No significant difference.
[c] ECV=external cephalic version.

[95]. Using chromosome 21-encoded mRNA, Oudejans et al. detected these placental-derived transcripts in maternal plasma [96]. These targets have implications of being gender- and polymorphism-independent fetal markers for non-invasive prenatal diagnosis. Potential application of circulating fetal RNA was demonstrated by a significant difference in plasma mRNA levels of CRH between preeclamptic and normal pregnancies [97]. Furthermore, using expression microarray technology, Tsui et al. established a systematic approach in generating new placental-derived mRNA markers for non-invasive gene expression profiling [98] (Fig. 1). Transcripts identified by this approach are pregnancy-specific, as shown by the clearance results (Fig. 2).

3.3. Stability of plasma RNA

With the well-known instability of RNA species, detection of circulating RNA was perhaps rather surprising. In this regard, it has been proposed that circulating RNA might be contained in apoptotic bodies, hence being protected from degradation by nucleases [99,100]. One line of empirical evidence was provided by Ng et al., who subjected plasma samples through filters with different pore sizes [101]. Results showed the presence of filterable and nonfilterable mRNA species in plasma. Tsui et al. further demonstrated the different stability of endogenous and

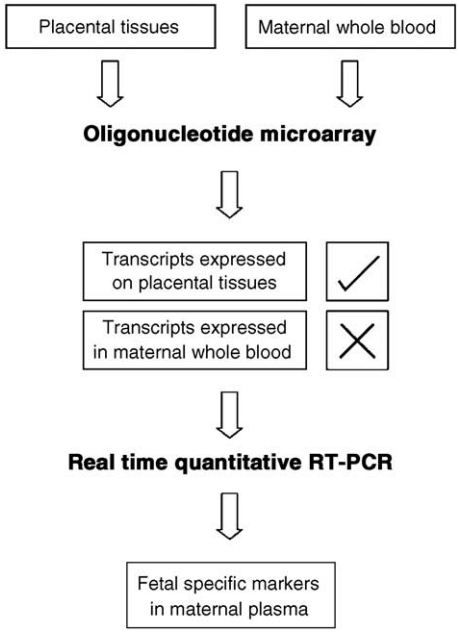

Fig. 1. Outline of strategy used for the systematic identification of pregnancy-specific placental expressed mRNA markers in maternal plasma. Paired placental tissues and maternal whole blood samples are collected and subjected to oligonucleotide microarray analysis. Transcripts with increased expression in the placental tissues relative to whole blood are selected and their detectability in maternal plasma and pregnancy specificity are evaluated by QRT–PCR on maternal plasma. Reproduced from: Tsui NBY, J Med Genet 2004;41:461–7 with permission from the BMJ Publishing Group.

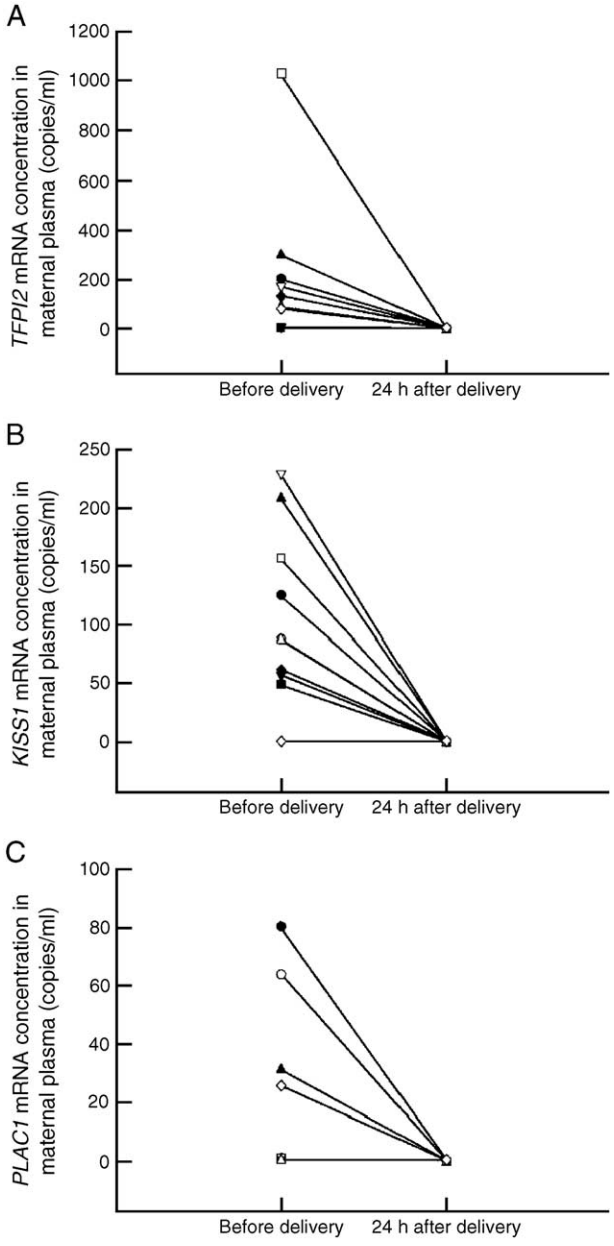

Fig. 2. Clearance of placental mRNA from maternal plasma after delivery. Concentrations of (A) *TFPI2* mRNA, (B) *KISS1* mRNA, and (C) *PLAC1* mRNA in maternal plasma before delivery and at 24 h after delivery were measured by QRT–PCR. Each line (symbol) represents one plasma sample obtained from one subject. Reproduced from: Tsui NBY, J Med Genet 2004;41:461–7 with permission from the BMJ Publishing Group.

exogenous RNA in plasma [102]. The stability of circulating RNA enables the RNA targets to become practical markers for non-invasive analysis.

4. Cell-free nucleic acids in urine

In addition to circulating nucleic acids in plasma and serum, DNA in urine represents another potential tool in molecular analysis.

For example, Eisenberger et al. detected microsatellite alterations identical to those found in the primary tumor in serum and urine of renal cancer patients [103]. Tumor-derived DNA was also detectable in urine, as well as plasma and serum, of patients who suffered from prostate carcinoma [104,105] and bladder cancer [106]. Zhang et al. demonstrated the detection of male donors' DNA in female renal transplant recipients using Y-chromosomal sequence as a marker for donor-derived DNA. Increase in urinary DNA concentration during acute rejection was observed and was followed by a rapid return to normal level with anti-rejection treatment [107,108]. The potential use of cell-free DNA in organ transplant monitoring was also illustrated in a study of detecting donor-specific DNA in the plasma of kidney and liver transplant recipients [109].

Botezatu et al. demonstrated that the kidney barrier is permeable to polymeric cell-free DNA by detecting male-specific sequences in the urine of females receiving male blood transfusion or carrying a male fetus [110]. Moreover, mutations of the K-ras gene were detected in the urine of patients with colon and pancreatic cancers. Su et al. further investigated the origin and size of cell-free DNA in body fluids by developing methods to enrich for non-cell-associated DNA fragments of between 150 to 250 bp [111]. Identical K-ras mutations were present in low molecular weight urine DNA and the corresponding tumor tissue DNA of colorectal carcinoma patients [112].

5. Development of diagnostic methods

With over five decades of development, important technical advancements have been made towards the study and diagnostic applications of circulating nucleic acids. Early methods such as radioimmunoassay could only detect nanogram quantities of DNA [4]. Such relative insensitivity might have contributed to the low diagnostic values of these assays. With the introduction of PCR, picogram quantities of DNA could be detected. The robustness of microsatellite analysis has been improved by fluorescence-based allelotyping techniques involving capillary electrophoresis [32]. In most of the studies on viral DNA detection in cancer patients, a positivity of over 50% was obtained. Quantitative real-time PCR further increased the sensitivity to over 90% [48,52]. In addition, detection of fetal DNA in maternal plasma by real-time PCR can achieve a close to 100% sensitivity and specificity for applications involving targets like Y-chromosome markers and the RhD gene [68]. However, effective anti-contamination measures should be strictly imposed since the sensitivity of quantitative PCR is high.

6. Rapid testing

Rapid testing involves fully automated systems in nucleic acid isolation, PCR mixture preparation, and rapid thermal

cycling profile, preferably with no post-PCR processing. As there are many parallels between the detection of human-derived plasma DNA/RNA and the detection of viral nucleic acids, we have cited below a number of studies aimed primarily for viral nucleic acid detection, but with applicability to the extraction and detection of human-derived plasma nucleic acids.

In a study to compare manual and automated (MagNA Pure LC) plasma nucleic acid isolation methods [113], Alp et al. observed a higher detection rate of viral RNA isolated from patients' plasma by the automated system (23/35, 65.7%) than by conventional manual method (20/35, 57.1%). When both of the methods showed a positive detection, significantly higher copy numbers were detected by the automated system.

Using the LightCycler technology on real-time quantitative PCR assays, Gueudin et al. quantified human immunodeficiency virus RNA (HIV-1 group O) in 40 out of 48 (83.3%) plasma samples [114]. Changes in viral load during treatment were observed in serial samples. The authors have claimed that despite the high initial cost of acquiring the device, the per-sample cost of this assay is low, making it suitable for use in endemic zones. In a study by Stocher et al., a detection system of DNA for 5 human herpes viruses in a single LightCycler run was successfully developed [115]. Viral DNAs were spiked into cerebrospinal fluid, serum or plasma. The detection limits were found at 500 or 250 viral DNA copies/ml, depending on the PCR assay and specimen type used. Another example of LightCycler-based real-time PCR is the quantitative detection of EBV DNA in unfractionated whole blood, serum, or plasma [116]. As stated by the authors, this approach is rapid, and the closed-tube system eliminates the risk of PCR product carryover contamination and the need for post-PCR processing. Due to the efficient heat conduction with glass capillaries, small reaction volumes, and air as heating medium, the time needed for each PCR cycle is reduced to 15–20 s. 20 to 30 min are all that are needed for a 30- to 40-cycle PCR run.

In the following studies, automated DNA extraction using the MagNA Pure extractor followed by LightCycler real-time quantitative PCR were employed. In a study on EBV DNA load monitoring in whole blood, peripheral blood mononuclear cells (PBMCs) and plasma, the use of whole blood and plasma for transplant patients were equally feasible and accurate in early diagnosis of PTLDs [117]. In the study of hepatitis B virus (HBV) DNA detection in plasma, the detection limit was 200 HBV DNA copies/ml [118]. In a follow-up study on the detection of human herpes virus DNA by Stocher et al., a set of LightCycler PCR assays complemented with a single multiple internal control, an approach which allows for monitoring sample adequacy, was employed [119]. This type of setup was found to be rapid, labor saving and suitable for the routine diagnostic laboratory.

Loop-mediated isothermal amplification (LAMP) is a rapid amplification method based on strand displacement DNA synthesis with high specificity and efficiency [120]. Detection limit has been reported to be down to a few copies of the target. Its specificity is conferred by the recognition of the target sequence by six independent sequences in the initial stage and four independent sequences during the later stages of the LAMP reaction, which produces a product of a stem-loop DNA structure. Equipment required for running the reactions includes a regular laboratory water bath or heat block, implying a relatively low setup cost. Detection of LAMP products is by gel electrophoresis or turbidity measurements [121]. During the LAMP reaction, a large amount of by-product in the form of pyrophosphate ion is produced. By measuring the turbidity of the reaction tube, real-time monitoring of the LAMP reaction can be achieved. When gel electrophoresis is used for detection, care should be taken to avoid carry-over and cross-contamination.

The LAMP method has been employed in two studies on detection of human herpesvirus (HHV) DNA in patients' whole blood and plasma samples. For HHV-6 detection, improved sensitivity from 50 copies per reaction to 25 copies per reaction was described. HHV-6 DNA was detected in the plasma samples collected in the acute phase but not in the convalescent phase, compared with the positive detection in whole blood samples in both phases. As a result, rapid diagnosis of active HHV-6 infection would be possible on plasma samples [122]. For HHV-7 detection, similar results were obtained with a 60-min LAMP reaction from plasma samples, which showed positive detections in acute phase but not convalescent phase [123].

7. Conclusions

The discovery of circulating nucleic acids has opened up new possibilities for non-invasive detection and monitoring of various disease conditions. In cancer patients, detection and quantification of circulating nucleic acids have shown promise for cancer diagnosis and prognosis. Maternal plasma analysis can be used to detect or exclude certain diseases of the fetus, and quantification of fetal nucleic acids has the potential to be used to screen for certain pregnancy-associated complications. Other applications include monitoring following trauma and stroke, when circulating DNA level is correlated with the severity.

With the rapid advancements in technologies, sensitivity and specificity have been improved for the detection of both cell-free DNA and cell-free RNA species. However, in this high throughput era, a robust assay system with high efficiency is of great importance. Introduction of new methods, such as fully automated systems for nucleic acid purification [113], together with rapid PCR systems [114] and non-PCR amplification strategies [120], will further facilitate high throughput molecular analysis. With such developments, the field of circulating nucleic acids will

likely undergo further expansion over the next few years and new clinical applications will likely be found.

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