Review

CIRCULATING NUCLEIC ACIDS AS A NEW DIAGNOSTIC TOOL

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Abstract: The discovery of circulating nucleic acids in the 1940s opened up new possibilities for the non-invasive detection, monitoring and screening of various human disorders. Several tumour markers that enable early cancer detection or tumour behaviour prediction have been detected in the plasma of cancer patients. Maternal plasma analysis can be used to detect certain fetal abnormalities, with the quantification of cell-free nucleic acids used to screen for several pregnancy-associated disorders. Some other applications are in transplant monitoring and graft rejection assessment, and in certain medical emergencies such as trauma and burn severity stratification. Many studies have yielded promising results in this field, but the techniques have yet to be applied in routine clinical practice. Large-scale studies using similar technologies and a broad spectrum of patients are still needed to verify the results of the various studies.

Key words: Circulating nucleic acids, RNA, DNA, Diagnostics, Cancer

INTRODUCTION

Circulating nucleic acids (CNAs) were first mentioned in the 1940s, when Mandel and Metais described their presence in the plasma of both patients and healthy individuals [1]. For a long time, studies on CNAs were mainly focused on autoimmune diseases such as rheumatoid arthritis or systemic lupus erythematosus [2, 3], where high levels of circulating DNA were detected in the serum of patients. In the 1970s, Leon *et al.* [4] demonstrated a decrease in the plasma CNA concentration in pancreatic cancer patients after chemotherapy. The importance of CNAs was recognized in the early 1990s, when Sorenson *et al.* described the presence of K-ras oncogene mutated products in pancreatic cancer patients [5] at approximately the same time as Anker *et al.* reported mutations of the N-ras gene in patients with myelodysplastic syndrome [6]. That was followed up by a number of publications reporting on CNA levels in different kinds of cancer and in non-cancer diseases such as stroke, sepsis, myocardial infarction, autoimmune diseases and other medical emergencies. Nowadays, it is generally known that the blood of cancer patients contains higher concentrations of DNA/RNA than the blood of healthy individuals. Thanks to the progress in polymerase chain reaction (PCR) diagnostics, it was possible to prove that the circulating DNA in cancer patients' plasma shows the same features as DNA isolated from a spectrum of tumour cells. The limitation on a more widespread usage of cell-free DNA in cancer diagnostics is that the mutations responsible for a given type of cancer must be known. Until recently, the persistence of circulating RNA in the blood had not been taken into consideration, but then several reports proved the presence in the serum of circulating RNA that is somehow protected from degradation by ribonucleases present in human plasma [7, 8]. Obtaining a plasmatic cell-free RNA sample is minimally invasive (simple vein punction), and further samples can be taken at any time. This enables the detection of possible progression or recurrence of the observed disease. Tumour RNA and DNA are released into the bloodstream in the early stage of cancer, which is why it is possible to use this for screening programs as well as early cancer diagnostics.

THE BIOLOGICAL BEHAVIOR OF CIRCULATING NUCLEIC ACIDS

The mechanisms of CNA release into circulation

There are several theories on how CNAs are released into the blood. In healthy individuals, CNAs enter the bloodstream after the apoptosis of nucleated cells such as lymphocytes or monocytes. In cancer patients, apoptosis has been considered as the possible source of cell-free nucleic acids. However, apoptosis is a mechanism supposedly suppressed in proliferating tumour cells, which weakens this theory. Another possible source of CNAs in the plasma are micrometastases, which are released actively into circulation, but this hypothesis is contradicted by the results of Sorenson and Chen [9-11], who measured higher amounts of DNA and RNA not corresponding with the number of tumour cells

presented in the blood. Therefore, the theory of micrometastases was also rejected. Another theory for the high amount of CNAs in the plasma of cancer patients is tumour necrosis [4, 12]. However, after radiotherapy, which is supposed to induce tumour necrosis, the amount of cell-free nucleic acids is paradoxically decreased in 90% of patients. This could be explained by the radiotherapeutic suppression of cancer cell proliferation [4]. Yet another possible source of nucleic acids in the plasma is the active release of these nucleic acids into circulation [9]. Another hypothesis takes into consideration the presence of DNase and RNase inhibitors in the serum of cancer patients. In the blood of healthy individuals, there is only a very small amount of cell-free DNA and RNA, probably because of the high activity of these enzymes. In the blood of cancer patients, a very low activity of these enzymes was detected, which supports this theory [13].

Forms of CNAs, and their stability and integrity
The nucleic acids released from cells are not "naked", but can be bound in complexes, or to the surfaces of cells via proteins [14]. Additionally, CNAs can complex with proteolipids [15] embedded in apoptotic vesicles [16]. Cell-free DNA is potentially a stable molecule that is found as fragments ranging in size from 500 bp up to over 30 kb [17]. Nucleosomes, which are complexes of DNA with protein components, can also be identified in the circulation. Holderrieder *et al.* detected these complexes in patients with cancer and with autoimmune diseases. They showed that measuring the level of nucleosomes in connection with other tumour markers can be useful in predicting the response to radiotherapy or chemotherapy [18].
Not many reports have focused on the possible mechanisms by which RNA is protected from plasma RNase activity. RNA is a very labile molecule that is easily degradated by the ubiquitous RNases. Recent reports have proved the presence of endogenous and exogenous cell-free RNA in the plasma. This suggests that circulating RNA is somehow protected against the activity of nucleases, probably by being contained in apoptotic bodies or bound to lipoprotein complexes [9]. Helfnawy *et al.* [19] passed plasma and serum samples through 0.2-μm filters, and found that the RNA concentrations were greatly decreased in the filtrate. He also proved that plasma endogenous RNA was stable at least for 3 h at room temperature in either whole blood or plasma before RNA extraction. However, the inherent plasma RNase activity immediately degraded the exogenous full-length RNA that was added (20 μg of total mouse spleen RNA). Additionally, commercial RNase inhibitors failed to protect the added RNA from degradation. In another experiment, Wong *et al.* used nasopharyngeal carcinoma patients as a model system for plasma RNA integrity analysis. The results showed a significant difference in the plasma RNA integrity between the two groups (49 untreated patients and 53 healthy controls; Mann-Whitney test, P = 0.024). The plasma RNA integrity was reduced in patients with nasopharyngeal carcinoma, and there was a linear correlation with the tumour stage [20].

Currently, exosomes are viewed as one of the significant sources of circulating nucleic acids. Exosomes are 40- to 100-nm diameter membrane vesicles of endocytic origin that are released by most cell types upon the fusion of multivesicular bodies with the plasma membrane, presumably as a vehicle for cell-free intercellular communication. Exosomes have now been identified in body fluids such as urine, amniotic fluid, malignant ascites, bronchoalveolar lavage fluid, synovial fluid, breast milk, saliva and blood. Recent findings that exosomes contain inactive forms of both mRNA and microRNA that can be transferred to another cell and be functional in that new environment have initiated many microRNA profiling studies of exosomes circulating in the blood. The exacerbated release of exosomes in tumor cells is evidenced by their increased levels in the blood during the late stage of the disease and the overexpression of certain tumor cell biomarkers, which suggests an important role for exosomes in diagnosis and biomarker studies [21-23].

APPLICATIONS OF CIRCULATING NUCLEIC ACIDS

Cancer diagnostics

The potential advantages of the detection and characterization of plasma circulating nucleic acids in cancer diagnostics were first reported by Sorenson and Anker [5, 6]. These striking findings suggested that circulating cell-free DNA could be used as a potential tool for cancer diagnostics. The first observations of the difference in the free-cell DNA concentration between normal individuals and cancer patients was reported by Leon *et al.* [4]. Most of the normal subjects have a low plasma DNA concentration in comparison to the increased DNA levels in many cancer patients. The level of plasma is not constant but fluctuates over time, and is not only connected with tumour growth. Therefore, single measurements of plasma DNA levels are not sufficient. Better information can be obtained via specific DNA marker detection. Mutations in oncogenes and tumour suppressor genes and microsatellite alterations are commonly detected in tumour tissues, and these changes are significant in carcinogenesis. Applying circulating plasma DNA in cancer testing depends on the accumulation of genetic and epigenetic changes such as chromosomal alterations, point mutations, microsatellite instability, and hypermethylation of tumour suppressor genes. To detect single tumour-associated mutations in the plasma or serum of cancer patients, allele-specific PCR is used. It allows the detection of a mutated sequence present in the plasma in a low concentration. Gormally *et al.* published the results of a large prospective study focused on the screening of bladder cancer. Their results showed that mutations in TP53 or KRAS2 could respectively be detected in the plasma circulating DNA of healthy subjects on average 20.8 months and 14.3 months before a cancer diagnosis. The presence of a mutation was not dependent on the total amount and concentration of the DNA extracted from the plasma: the geometric mean concentration in samples with a TP53 or KRAS2 mutation was 28 ng/mL [24]. Chromosomal

rearrangements are another class of events that can occur in neoplasia. There have been several articles reporting chromosome damage in workers exposed to certain carcinogens [25, 26]. Microsatellite instability, particularly loss of heterozygosity (LOH), has been observed both in tumour tissue itself and in the corresponding circulating DNA. Serum LOH detection has been shown to be a good marker in predicting disease outcome and the therapeutic response in malignant melanoma [27] or breast cancer [28]. Besides genetic alterations, tumour-associated epigenetic alterations have also been reported in the plasma or serum of cancer patients. DNA methylation is a physiological process that is active in healthy cells, but a growing number of human diseases are also associated with aberrant DNA methylation. This epigenetic DNA modification can result in gene silencing without changing its coding sequence. Methylation of the CpG islands in the promoter region of the tumour suppressor genes (TSG) has been suggested to trigger local gene silencing. Methylation of certain TSGs has been described as an early event in cancerogenesis, and has opened up the possibility of early cancer screening with circulating methylated tumour DNA. This process was identified in liver [29], lung [30] and breast cancer [31] and in solid tumours such as head and neck squamous cell carcinomas [32], and colorectal [33, 34], oesophageal [35] and ovarian cancer [36].

The first study associating circulating RNA in the serum as a potential tumour marker was reported by Wieczorek *et al*. That study reported an association between the presence of the RNA-proteolipid complex and the tumour response to therapy. The complexes disappeared approximately 2 days after tumour removal and were not detected in benign tumours [37]. In the case of neoplasia, circulating RNA has been found to be a more sensitive marker than tumour-derived circulating DNA. During the last ten years, there has been a great leap forward in detecting and testing isolated cell-free RNA for different tumour-related transcripts [38, 39], telomerase components [40] or viral RNA transcripts. Tumour-associated RNA was shown to be detectable in the serum or plasma of patients with breast, liver and lung cancer [41], colorectal cancer [42], follicular lymphoma [43], prostate cancer [44], malignant melanoma [45], hepatocelular carcinoma [46], oesophagel carcinoma, and head and neck squamous cell carcinoma [47].

A very limited number of studies have attempted to investigate the feasibility of identifying mRNAs that are potentially predictive of the response to therapy. Some studies focused on Her-2 and hnRNP-B1 mRNA in lung cancer [40] and PSMA and CEA mRNA in prostate cancer [48]. Demonstrating the presence of tumour-associated RNA transcripts in the plasma or serum provides not only new targets for cancer detection but also opens up the possibility for wider gene expression profiling of different types of cancer. A recently published study by Li *et al.* evaluated the serum transcriptome using gene expression profiling in patients with oral squamous cell carcinomas. He identified more than 300 differentially expressed genes in the serum of cancer patients in comparison to healthy individuals [49]. Several other studies followed using breast [50] or

colon cancer [51] as proof of concept for the disease. According to the latest discoveries in this area, the most promising tool seems to be global transcriptome profiling and predicting the panel of markers involved in individual steps of carcinogenesis.

MicroRNAs in the circulation are currently of immense interest. MicroRNAs are functional, 22 nt, non-coding RNAs that negatively regulate gene expression. Accumulating evidence suggests that microRNAs play important roles in many human cancers. They are pivotal regulators of diverse cellular processes including proliferation, differentiation, apoptosis, survival, motility and morphogenesis [52, 53]. In the initial studies, patients with small cell lung carcinoma and head and neck tumours were analyzed, and other groups made similar observations on tumours of the pancreas [54] and prostate [55], and in colon adenocarcinoma [56], lung cancer [57] and head and neck squamous cell carcinoma [58].

Even before cell-free nucleic acids became popular as a target for tumour detection, the presence of cell-free viral nucleic acids had been described. The association of a viral infection and the initiation of certain types of cancer makes the detection of viral nucleic acids an interesting potential method. There are several pieces of evidence for the presence of Epstein-Barr virus (EBV) DNA in the blood of nasopharyngeal carcinoma patients [59, 60] and for the presence of Human Papilloma virus (HPV) DNA in patients with cervical cancer [61] or head and neck squamous cell carcinoma [62].

Prenatal diagnostics

Routine prenatal diagnostic procedures including chorionic villus sampling (CVS), amniocentesis and cordocentesis include the risk of fetal loss, which is statistically around 1% depending on the method performed. The discovery of cell-free nucleic acids in the plasma and serum provides possibilities for non-invasive prenatal diagnostics. Lo *et al.* were the first to report the existence of circulating DNA in maternal plasma by detecting the Y-chromosome DNA sequence in maternal plasma in 1997 [63]. With the developments in detection and improvements in the sensitivity and specifity of these methods, maternal plasma proved to be useful for detecting chromosomal aneuploidies, sex-linked disorders [64], fetal Rhesus D factor and certain endocrine [65] and neurological disorders. Alterations in the quantity of circulating placental-derived RNA in the maternal blood are associated with many pregnancy-related disorders such as preterm delivery or preeclampsia [66], fetal-maternal hemorrhage [67] and polyhydramnios. Successful prenatal diagnosis of Mendelian disease involves the detection of paternally inherited alleles or mutations in the maternal plasma. In the case of autosomal dominant diseases, a positive detection signifies the inheritance of the disease in the fetus. Diseases in which prenatal testing has been successfully achieved by fetal DNA maternal plasma include achondroplasia [68], myotonic dystrophy [69] and Huntington's disease [70]. On the other hand, the opposite interpretation applies in cases of autosomal

recessive diseases. Prenatal exclusion of congenital adrenal hyperplasia [71], cystic fibrosis [72] or beta-thalassaemia [73] is also possible.

The recent demonstration of the presence of placenta-derived fetal RNA in maternal plasma has opened up better opportunities for non-invasive prenatal investigation. Circulating fetal RNA analysis could in principle be applied to all pregnancies without the limitations of fetal gender or polymorphisms between the mother and fetus. The use of fetus- or disease-specific circulating RNA markers would greatly increase the number of markers that can be used for prenatal monitoring. With the advances in microarray technology, new placenta-derived plasma RNA markers can be widely identified. These newly identified placental transcripts were easily detectable in the maternal plasma and were pregnancy-specific. Over the past couple of years, there have been encouraging reports on the detection and possible clinical application of circulating fetal RNA. Placental-derived RNA has been shown to be easily detectable in maternal plasma during pregnancy, and rapidly cleared after delivery. Such observations suggest that the placenta is an important organ for releasing fetal RNA into the maternal plasma. Non-invasive prenatal gene expression profiling of the placenta has also been demonstrated to be feasible via analysis of the circulating placental RNA in the maternal plasma. After a decade of work, the theoretical and practical feasibility of prenatal fetal chromosomal aneuploidy detection by plasma nucleic acid analysis has been demonstrated in studies using small sample sets [74, 75]. Larger-scale cohort studies will be needed to validate these initial observations. If these larger-scale studies prove to be successful, it is expected that molecular non-invasive prenatal diagnosis of the major chromosomal aneuploidies and certain Mendelian diseases could become a routine practice in the near future.

Other diseases, emergencies and transplant monitoring
As described above, the first publications about circulating nucleic acids reported on the presence of CNAs in different autoimmune disorders such as systemic lupus erythematosus or rheumatoid arthritis [76]. Many authors predicted the level of cell-free nucleic acids to be connected with cell death, even though the origin of these acids in the circulation has yet to be proved. As cell necrosis occurs in many medical emergencies, plasmatic cell-free DNA or RNA levels were studied in acute cases such as myocardial infarction, stroke and adult respiratory distress syndrome, and in patients with physical trauma and burns. Rainer *et al.* showed plasma DNA levels to be a useful marker for risk stratification in stroke patients [77]. In another study, by Chang *et al.*, the correlation between plasma circulating DNA levels and prolonged myocardial ischemia was proven. This could be used for myocardial infarction diagnostics next to other blood markers such as the troponin, CK-MB. Rainer *et al.* published several reports about higher plasma DNA and mRNA levels in trauma patients [78]. Recent reports focused on the quantification of circulating RNA in burn patients. Fox *et al.* reported higher expression of mRNA and higher levels of cell-free DNA in the first 48 h following burn injury. Plasma RNA and DNA

levels are related to the severity in terms of the percentage of the total body surface area (TBSA) that was burnt [79].

Measuring the circulating DNA and organ-specific RNA has also been found to be useful in situations other than oncology, fetal medicine and emergency cases. These include several autoimmune diseases and diabetes mellitus. Diabetic retinopathy, which is a serious complication of diabetes, is difficult to detect. Recent studies reported that the mRNA for rhodopsin in the circulation was significantly higher in patients with diabetic retinopathy, and preliminary results seem to indicate a promising approach for the detection of long-term diabetic retinopathy complications [80, 81]. Analysis of circulating DNA in the plasma could also provide a useful biomarker in sickle cell disease (SCD), in view of the increased cell turnover through chronic ongoing haemolysis, recurrent vaso-oclusion and inflammation. Vasavda *et al.* reported the correlation between DNA levels in the plasma and C-reactive protein levels and total white cell counts in SCD patients [82].

Another field of medicine that may benefit from the further study of CNAs is transplantation medicine. Even with advances in immunosuppressive treatment, graft rejection is still a severe problem in human allotransplantation. Progresses in circulating nucleic acid study forced researchers to search for donor-derived DNA and RNA in the recipient's circulation. Thusfar, donor-derived DNA has been isolated from the blood of liver, kidney, pancreas and bone-marrow transplant recipients [83].

METHODS OF DETECTING CIRCULATING NUCLEIC ACIDS IN THE CIRCULATION

The first trials on CNA isolation began in the 1940s, but the discovery of their broader significance and further development in this field is dated to the 1990s. Cell-free nucleic acids can be isolated by phenol-chloroform extraction based on the original method developed by Chromczynski and Sacchi [84] or by using commercially available kits. Consistent amounts of CNAs have been obtained using amounts of plasma or serum in the range of 100-500 μl with resuspension of the isolated DNA/RNA in an elution buffer in the range of 15-100 μl, depending on the kit used. Helfnawy *et al.* demonstrated several isolation methods for cell-free RNA and compared their efficiencies depending on the used protocols and reagents [24]. For the extraction, nine commercial kits were used: Rneasy Mini Kit (Qiagen), QIAmp Viral RNA Mini Kit (Qiagen), SV Total RNA Isolation system (Promega), Eppendorf Perfect RNA Eukaryotic mini reagent set (Brinkman Instruments Inc.), Magna Zorb DNA Mini-Prep Kit (Cortex Biochem) and TriBD Reagent (Sigma). There was an added protocol using modified extraction based on the original method of Chromczynski and Sacchi (1987). The best efficiency was achieved using the methods based on precipitation: GIT-phenol extraction and TriBD Reagent. As mentioned before, one of the disadvantages of the detection of circulating DNA/RNA is the low

amount available for further analysis. Previous experiments worked on the idea
of concentrating larger volumes of plasma before RNA extraction using different
approaches such as concentrating the plasma RNA with spin filters or in
a concentrator. Nowadays, these methods seem rather obscure in comparison
with newly established methods of detection that can detect even a single
molecule in a sample. Earlier, sub-nanogram levels of plasma CNA could not be
detected using a radioimmunoassay (P32DNA) [4], but now, CNA levels down
to picogram levels can be detected using PCR/RT-PCR. Quantitative real-time
PCR further increased the sensitivity of detection of cell-free tumour- or fetal-
derived DNA/RNA to over 90% sensitivity [85], and in some cases, such as the
Y-chromosome in maternal blood or the RhD fetal status, close to 100%
sensitivity [86]. In the last 10 years, there have been several studies concerned
with the RT-PCR-based detection of breast, lung, colon, liver and bladder
cancer, malignant melanoma [45, 87], or head and neck squamous cell
carcinomas [49]. The main disadvantage of these approaches remains that in all
cases only a single marker or a panel of single markers was tested. This
markedly influenced the sensitivity and specificity of the approach. Using
microarray analysis for the detection of the whole human serum transcriptome is
a current promising approach. Li *et al.* was one of the first to target the serum
transcriptome in patients with head and neck squamous cell carcinoma, using
high density oligonucleotide microarrays [49, 88]. Recent studies have been
dominated by three new detection methods: nucleic acid mass spectrometry,
digital PCR and second generation DNA sequencing. These methods can be
used in prenatal diagnostics of chromosomal aneuploidies and diagnostics of
several types of tumours [89]. In the case of prenatal testing, the major problem
is that cell-free DNA from the maternal plasma mainly consists of maternal
DNA sequences (more than 95%). For mass spectrometry, alternate strategies
have been developed using single nucleotide polymorphisms (SNPs): for
trisomy21, the analysis of PLAC4 [90], for trisomy18, the analysis of maspin
[91]. Digital PCR is based on quantification via the use of limited dilution and
Poisson statistics. For more complex analyses, it is necessary to use upgraded
systems, such as emulsion PCR or microfluidic devices. While emulsion PCR
provides millions of individual microreactions [92], digital PCR on microfluidic
devices enable the performance of highly parallel analyses in a single PCR step
[93]. Preamplification can increase the sensitivity in certain applications,
especially in the case of fetal DNA analysis in maternal plasma. Some reports
documented that the distinction could be observed even when the aneuploid
material was 10% of the examined portion [94]. The high-throughput shotgun
sequencing is a highly efficient method for the non-invasive detection of fetal
aneuploidy. Fan *et al.* successfully identified all nine cases of trisomy 21, two
cases of trisomy 18 and one case of trisomy 13 in a cohort of 18 normal and
aneuploid pregnancies [95]. The methods of isolation and quantification of
plasma/serum CNAs are crucial in analysing the data. We still need to
standardize the techniques of isolation, detection and quantification of cell-free

DNA/RNA. The results still vary considerably depending on the isolation kit used, the detection and quantification method, and the means of data analysis.

CONCLUSIONS

The study of circulating nucleic acids is a relatively new but rapidly expanding field of medical research. With the increasing demand for non-invasive approaches in the monitoring of cancer, prenatal condition and transplant rejection, circulating nucleic acids have been shown to be a promising tool (see Fig. 1). The development of circulating mRNA detection has yielded an approach for non-invasive gene expression profiling. With the advances in technology and detection methods, the sensitivity, specifity and overall outcome have been improved for the detection of both circulating DNA and RNA specimens. However, there are still challenges for the standardisation of these procedures.
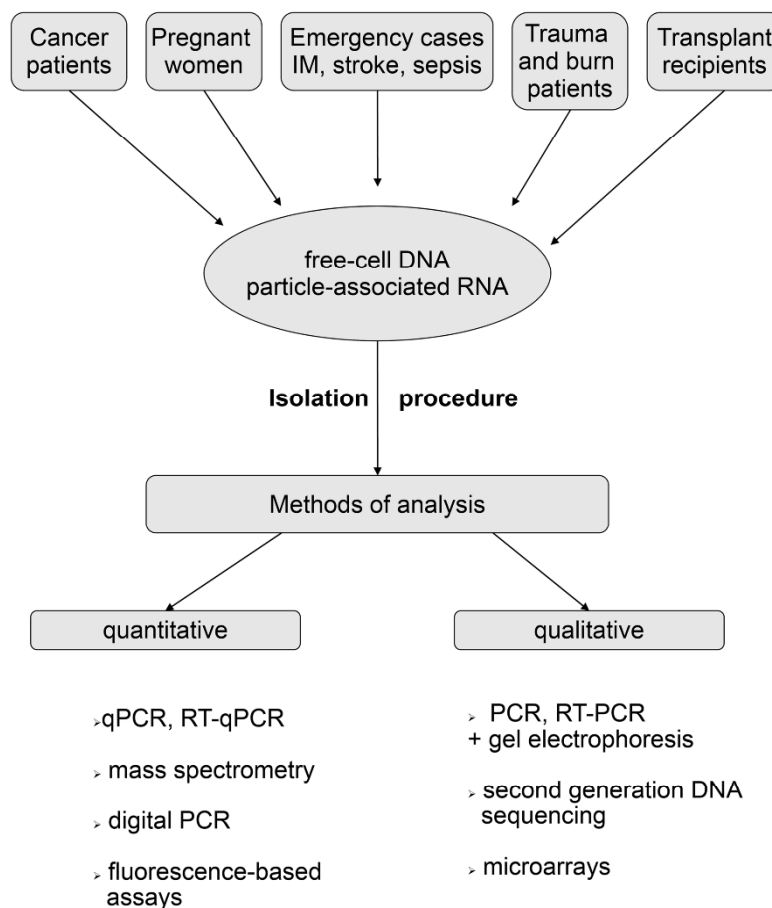

Fig. 1. A diagram of the methods of detecting circulating nucleic acids in the blood.

Currently, there is no consensus on the sample type, sample amount and sample preparation protocols for further analysis of circulating nucleic acids. Preanalytical factors including sample taking, storage, and the used chemicals, such as anticoagulants or Trizol solution, seem to affect the quantity and quality of the circulating nucleic acids. To elaborate on these issues, we need to better understand the biological behavior, release and protective mechanisms of cell-free nucleic acids in the blood. Overall, it seems that after certain developments in this field, circulating nucleic acids will become a very useful tool for many clinical applications.

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