

Circulating Nucleic Acids in Plasma and Serum

Recent Developments

R. SWAMINATHAN AND ASIF N. BUTT

Department of Chemical Pathology, St Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, UK

ABSTRACT: Nucleic acids (DNA and RNA) have been detected in plasma, serum, urine, and other body fluids from healthy subjects as well as in patients. The ability to detect and quantitate specific DNA and RNA sequences has opened up the possibility of diagnosis and monitoring of diseases. With the recent developments in the field of circulating nucleic acids the application in the diagnostic field has increased. The recent discovery of epigenetic changes in placental/fetal DNA and the detection of fetal/placental-specific RNAs have made it possible to use this technology in all pregnancies irrespective of the gender of the fetus. With the application of mass spectrometry and other techniques to this field, it is now possible to detect very small amounts of specific DNA in the presence of excess of other nonspecific nucleic acids (e.g., detection of mutations in fetal DNA in the presence of excess of maternal DNA). Circulating nucleic acids have now been shown to be useful in other conditions, such as diabetes mellitus, trauma, stroke, and myocardial infarction. In oncology, detection and monitoring of tumors is now possible by the detection of tumor-derived nucleic acids. In spite of these advances questions regarding the origin and biologic significance of circulating nucleic acids remain to be answered. Furthermore preanalytical and analytical aspects of this field remain to be standardized.

KEYWORDS: circulating DNA; RNA; oncology; prenatal diagnosis; transplantation

INTRODUCTION

The fourth international conference on circulating nucleic acids in plasma/serum was recently held in London, England at King's College. The conference was held at the Franklin–Wilkins Building where, in the MRC Bio-

Address for correspondence: R. Swaminathan, Department of Chemical Pathology, 5th Floor, North Wing, St Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, UK. Voice: +44-20-7188-1285; fax: +44-0-20-7928-4226.
e-mail: r.swaminathan@kcl.ac.uk

Ann. N.Y. Acad. Sci. 1075: 1–9 (2006). © 2006 New York Academy of Sciences.
doi: 10.1196/annals.1368.001

physics unit, Rosalind Franklin and Maurice Wilkins described the crystallographic structure of DNA. The aim of the fourth conference was to bring together scientists and clinicians interested in this field to present and to discuss the latest advances in circulating nucleic acids. Areas of discussion included the biology of nucleic acids in circulation, circulating nucleic acids in fetal medicine, oncology, diabetes, and other diseases, as well as analytical and preanalytical aspects. The conference was attended by over 200 delegates and there were 20 symposium presentations, 31 oral presentations, and 79 poster presentations.

In 1947 Mendel and Metais discovered DNA in plasma and serum.¹ This was before Watson and Crick described the helical structure of the DNA. Mandel and Metais, using a simple perchloric acid precipitation method, showed the presence of nucleic acids in healthy subjects as well as in ill patients. Interestingly they reported high amounts of DNA in serum from pregnant women. This discovery of nucleic acids in plasma and serum was forgotten until the 1960s, when it was shown that patients with systemic lupus erythematosus (SLE) had higher levels of circulating DNA.² Further work in 1970 showed that increased levels of DNA in circulation could be detected in other diseases, such as rheumatoid arthritis, glomerular nephritis, pancreatitis, inflammatory bowel disease, and hepatitis.³ This was followed by the discovery of higher concentration of circulating DNA in patients with cancer compared to patients without cancer.⁴ Furthermore, it was shown that concentration of circulating DNA was higher in patients with metastases compared to patients with localized disease and that the circulating DNA concentration decreased by up to 90% following radiotherapy.⁴ The cellular origin of the extracellular DNA was not determined until the 1980s, when Stroun and Anker showed that circulating free DNA was derived from tumor cells. They showed that certain characteristics of tumor DNA were also present in circulating DNA.⁵ Further work in the 1990s showed that oncogene mutations, loss of heterozygosity (LOH), and microsatellite shifts can be found in the plasma and serum matching those occurring in primary tumors.⁶ The next major breakthrough in the field of circulating nucleic acids was the discovery of fetal DNA in maternal plasma by Lo *et al.*⁷ This opened up a new field of investigation of noninvasive prenatal diagnosis. Measurement of circulating fetal DNA in maternal plasma has been used as a noninvasive method for fetal gender determination, assessing chromosome ploidy and monitoring various pregnancy-related complications. A summary of the highlights of the fourth international conference on circulating nucleic acids in plasma/serum is presented below.

Biology of Circulating Nucleic Acids

In a series of elegant experiments Stroun and co-workers showed that after three generations of grafting between two varieties of eggplant, hereditary

characteristics could be transmitted between plants.⁸ They proposed that DNA circulates in the free form, which can enter somatic and reproductive cells. Further experiments showed that the DNA released from living cells was accompanied by DNA polymerase and that there seemed to be preferential release of newly synthesized DNA.⁹ These early studies showed the presence of circulating nucleic acids in the free form and that they have the potential to initiate synthesis of RNA.¹⁰ One of the functions of circulating DNA may be to act as a messenger between cells and tissues within the organism.

Anker *et al.* suggested that circulating DNA may be involved in the immune response. They showed that when nude mice were injected with DNA released from T lymphocytes, which had previously been exposed to inactivated polio virus, they produced neutralizing antibodies.¹¹ Furthermore, mice injected with DNA released by human T cells were shown to produce antibodies carrying human allotypes as shown by neutralization by antiallotype serum. These studies suggest circulating DNA may have a role in the immune response. However, at this time it is difficult to define the exact role of circulating DNA in the immune response.

In addition to DNA it is known that RNA can be detected in the circulation. It is now well documented that RNA can be detected in plasma, serum, and other body fluids, as well as from cell-free supernatants of *in vitro* cultivated cells. The RNA appears to be complexed with other molecules, making it resistant to digestion by RNase.¹² The mechanism by which RNA is released into the circulation is still not clear. Possible mechanisms suggested are an active process and a nonspecific release from apoptotic or necrotic cells. Like DNA, RNA in the circulation can also be taken up by other cells. Over the past several years messenger RNA detected in the circulation includes fetal genes in the circulation of pregnant women, housekeeping genes in healthy persons as well as in patients with disease, and genes overexpressed in a variety of tumors.¹³ In spite of such a large literature on circulating RNA, the biological significance of circulating RNA is not yet known. Measurement of circulating messenger RNA has been used in the field of oncology as possible tumor markers and in the field of fetal medicine to detect fetal abnormalities and pregnancy complications. However, the full potential of measurement of circulating RNA in circulation is yet to be realized and this will depend on new methods and further diagnostic work.

Nucleic Acids in Fetal Medicine

Since the discovery of fetal nucleic acids in maternal plasma there have been a large number of publications on the possible applications and the significance of fetal nucleic acids. However, there are very few studies on the factors that influence cell-free DNA levels in normal pregnancies. Bianchi and co-workers have examined in detail the effect of gestational age, race, parity,

smoking history, and type of conception (natural or assisted). They showed that gestational age is a significant factor affecting fetal DNA levels. Fetal DNA levels increase by about 21% per week in the first trimester. Smoking and maternal age have no influence on fetal DNA concentrations; however, there was a significant inverse correlation between maternal weight and fetal DNA levels.¹⁴ There was a difference in the fetal DNA levels according to the type of conception.¹⁵ Using three-dimensional ultrasound examination the relationship between placental volume and cell-free DNA levels were examined and no association was found between these variables.¹⁶

Most of the studies on fetal DNA are based on the detection of Y chromosome signal in the presence of a male fetus. The progress in the application of circulating nucleic acids in fetal medicine is hampered by the lack of a universal fetal DNA marker. Lo and co-workers have recently shown the potential value of fetal epigenetic markers. In an important study, Poon *et al.*¹⁷ showed that a region of DNA, which is methylated, if inherited from the father, and unmethylated, if inherited from the mother, could be used to differentiate fetal origin of DNA in circulation. These workers then went on to show that the maspin gene is hypomethylated in the placenta, but hypermethylated in maternal blood cells.¹⁸ Hypomethylated maspin sequences were shown to be present in the plasma of pregnant women and this was rapidly cleared after delivery. The concentration of hypomethylated maspin in the circulation was elevated nearly six times in the plasma of women suffering from pre-eclampsia, showing that hypomethylated maspin could be a very useful fetal marker. This has opened up a new field of noninvasive prenatal diagnosis that can be applied to all pregnancies.

Another approach to detect fetal cell-free DNA in maternal plasma is to examine the size.¹⁹ Li *et al.* have shown that fetal cell-free DNA is smaller than comparable maternal cell-free DNA molecules.¹⁹ They extracted DNA from maternal plasma and subjected it to gel electrophoresis. Following gel electrophoresis they carefully sliced discreet fragments of different sizes and examined the fragments for fetal DNA using the SRY locus. This showed that the majority of fetal DNA had a molecular size of less than 300 bp whereas most of the maternal DNA was greater than 300 bp. This finding suggests that fetal cell-free DNA could be enriched on the basis of its size. The applicability of this to clinical diagnosis was shown by the ability to detect achondroplasia and β -thalassemia prenatally.²⁰ By isolating the cell-free DNA from maternal plasma according to size and using an allele-specific real-time PCR-based approach, they demonstrated the feasibility of detecting β -thalassemia in maternal plasma.

Fetal DNA has been successfully used in the fetal blood group genotyping. Daniels *et al.* have shown that by using fetal DNA in maternal plasma Rh status of the fetus can be successfully determined. Using real-time quantitative PCR they were able to detect RHD exons 4, 5, and 10.²¹ By incorporating SRY and in RHD-negative females eight biallelic polymorphisms, they provide an

internal positive control to overcome inadequacies in PCR amplification. This technique is greater than 99% accurate in detecting Rh status of the fetus according to experience from the National Blood Group Reference Laboratory in Bristol and Sanquin Laboratory in Amsterdam. Prospects for the detection of other blood types, such as RHc, Rhe, K, FY look very promising.²²

Nucleic acids in maternal plasma are also useful in the detection of maternal complications, such as pre-eclampsia and intrauterine growth retardation (IUGR). In a longitudinal study of 130 pregnant women using total DNA (β -globin) and fetal DNA (SRY), Butt and co-workers were able to show that higher levels of β -globin and SRY genes were detected in those pregnant mothers who went on to develop IUGR or pre-eclampsia, showing that circulating nucleic acids may be a useful way of predicting abnormal outcome of pregnancy. In addition to fetal DNA, fetal RNA could be detected in maternal plasma and this has opened up another approach for noninvasive prenatal diagnosis. Measurement of circulating fetal RNA has the advantage that it can be applied to all pregnancies irrespective of the fetal gender or polymorphisms between mother and fetus. Recent studies have shown that fetus-specific circulating RNA has greatly increased the number of markers that could be used for prenatal diagnosis and monitoring.²³ Using microarray technology, Tsui *et al.* identified new placental-derived plasma RNA markers. It was shown that the concentration of mRNA for corticotrophin-releasing hormone (CRH) is increased 10-fold in pre-eclamptic women, which was related to the severity of pre-eclampsia.²⁴

An important aspect that has emerged from these studies on the use of fetal DNA in maternal plasma for prenatal diagnosis is the lack of assay standardization, wide differences in sample collection and preparations, as well as the lack of a proper external quality assurance scheme. Recently, the special noninvasive advances in fetal and neonatal evaluation network (SAFE) was established with funds from the European Union with the aim of implementing routine cost-effective, noninvasive prenatal diagnostic methods.²⁵

Nucleic Acids in Oncology

Development of malignancy is associated with many genetic and epigenetic alterations. These include allelic loss, microsatellite instability, point mutations, amplification, translocation, and gene promoter region hypermethylation. These genetic and epigenetic alterations in the tumor have also been detected in circulating DNA. The detection of the circulating nucleic acids has provided a potential tool for the early diagnosis, monitoring, and prognosis of cancer patients. Studies showing the potential application of circulating nucleic acid have been done for many tumors, including gastrointestinal, breast, lung, head and neck, urological, gynecological, and skin. Hoon's group has shown that in melanoma patients, the outcome of

the disease could be predicted by the measurement of loss of heterogeneity in many different genes.²⁶ This is especially true in patients in late stages of melanoma. Similarly, the detection of methylation status of circulating DNA was also shown to be a predictor of outcome in melanoma subjects.²⁷ Sidransky's group have shown that aberrant DNA methylation can be detected in the serum and plasma of lung cancer patients. Using tumor and matched serum and plasma DNA, these investigators examined aberrant methylation for several gene promoters and showed that in the majority of lung cancer patients hypermethylation can be detected.²⁸ This approach could therefore be used for early detection and surveillance of lung cancer. Recent studies from Woll's group suggest that LOH and promoter methylation can be detected not only in lung cancer patients, but also with increasing frequency in smokers and those with dysplastic changes.²⁹ This suggests that circulating DNA may be able to identify individuals who are at risk of developing lung cancer.

Nucleosomes, which are complexes of DNA with several protein components, can also be detected in the circulation. Holdenrieder *et al.* have identified circulating nucleosomes in patients with cancer and in patients with autoimmune disease. They showed that measurement of nucleosomes together with other tumor markers can be useful in predicting the outcome of chemotherapy and radiotherapy.³⁰

Nucleic Acids and Other Diseases

Measurement of circulating DNA and organ-specific RNA has also been found to be useful in situations other than oncology and fetal medicine. These include trauma, stroke, diabetes mellitus, and autoimmune disease. Diabetic retinopathy, which is a serious and major complication of diabetes, is difficult to detect. Recent studies have shown that messenger RNA for rhodopsin in the circulation was significantly higher in patients with diabetic retinopathy and preliminary results suggest that it may be a useful test for the prediction of long-term diabetic retinopathy complication.³¹ In patients presenting to the emergency department, plasma nucleic acid concentration was found to be elevated within an hour of the injury and the concentration of DNA is related to the severity of the injury.³² Furthermore, measurement of DNA at the patient's time of admission was able to predict the outcome in terms of organ failure, acute lung injury, acute respiratory syndrome as well as death. Similarly, in patients presenting with stroke, measurement of β -globin DNA concentration was found to be higher and this was a good predictor of death as a result of the stroke.³³ In patients with chest pain (acute coronary syndrome) plasma DNA concentration was elevated and the degree of elevation was related to the extent of injury; it was highest in those patients who died within 2 years.

The measurement of circulating nucleic acids is also useful in the detection of transplant rejection. Despite HLA matching and the introduction of new immunosuppressive drugs, acute allograft rejection is one of the major

complications of transplantation. Accurate and early diagnosis of transplant rejection remains a stumbling block to the success of organ transplantation schemes. Measurement of donor-specific DNA or measurement of mRNA for granulysin and FOXP3 in urine has been found to be a useful predictor of kidney transplant rejection.^{34,35}

Methodology Aspects

Analysis of circulating nucleic acids in plasma and serum is conventionally done by real-time PCR because of the low copy number of the target DNA and RNA. The presence of a vast excess of background DNA and RNA makes this a difficult task. The application of MALDI-TOF mass spectrometry to PCR has enabled the detection of nucleic acids with great sensitivity. Ding *et al.* have developed a single allele primer extension reaction (SABER) method to achieve one base specificity and with the combination of the SABER method and MALDI-TOF mass spectrometry, they were able to show noninvasive prenatal diagnosis of β -thalassemia, where there is a single base point mutation.^{36,37} Cirigliano has approached the problem of low sensitivity by the use of quantitative fluorescent PCR (QF-PCR). QF-PCR is based on the application of highly polymorphic chromosome-specific short tandem repeats using fluorescent primers, and the products are analyzed using an automated DNA sequencer.³⁸ These workers have introduced this technique for the prenatal diagnosis of several disorders. On the basis of experience several thousand samples with, they show that this method is accurate and could reduce the need for amniocentesis or chorionic villus sampling. The main advantages of QF-PCR are its accuracy, speed, automation, and low cost.

CONCLUSIONS

Since the discovery of nucleic acid in plasma and serum, the field has advanced and has produced many exciting new developments. Plasma DNA is already in routine use for fetal blood group typing in many countries. With the rapid development in molecular biology techniques, the application of circulating nucleic acids in diagnostics, especially in cancer and prenatal diagnosis, is expected to be revolutionized. Some of these new developments and new insights into the biology of circulating nucleic acids were presented at the CNAPS IV conference, and are presented in this volume. In the next few years further developments will determine the clinical applications of circulating nucleic acids in diagnosis and monitoring of diseases.

REFERENCES

1. MANDEL, P. & P. MÉTAIS. 1948. Les acides nucléiques du plasma sanguin chez l'homme. C. R. Acad. Sci. Paris **142**: 241–243.

2. TAN, E.M. *et al.* 1966. Deoxyribonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. *J. Clin. Invest.* **45**: 1732–1740.
3. KOFFLER, D. *et al.* 1973. The occurrence of single-stranded DNA in the serum of patients with systemic lupus erythematosus and other diseases. *J. Clin. Invest.* **52**: 198–204.
4. LEON, S.A. *et al.* 1977. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res.* **37**: 646–650.
5. STROUN, M. *et al.* 1989. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* **46**: 318–322.
6. NAWROZ, H. *et al.* 1996. Microsatellite alterations in serum DNA of head and neck cancer patients. *Nat. Med.* **2**: 1035–1037.
7. LO, Y.M.D. *et al.* 1989. Prenatal sex determination by DNA amplification from maternal peripheral blood. *Lancet* **2**: 1363–1365.
8. STROUN, M. *et al.* 1963. Modifications transmitted to the offspring, provoked by heterograft in *Solanum melongena*. *Arch. Sci. Genève* **16**: 1–21.
9. STROUN, M. & P. ANKER. 1971. Bacterial nucleic acid synthesis in plants following bacterial contact. *Mol. Gen. Genet.* **113**: 92–98.
10. GAHAN, P.B. *et al.* 2003. Evidence that direct DNA uptake through cut shoots leads to genetic transformation of *Solanum aviculare* Forst. *Cell Biochem. Func.* **21**: 11–17.
11. ANKER, P.D. *et al.* 1984. Nude mice injected with DNA released by antigen stimulated human T lymphocytes produce specific antibodies expressing human characteristics. *Cell Biochem. Func.* **2**: 33–37.
12. ROSI, A. *et al.* 1988. RNA-lipid complexes released from the plasma membrane of human colon carcinoma cells. *Cancer Lett.* **39**: 153–160.
13. LLEDO, S.M. *et al.* 2004. Real time quantification in plasma of human telomerase reverse transcriptase (hTERT) mRNA in patients with colorectal cancer. *Colorectal Dis.* **6**: 236–242.
14. WATAGANARA, T. *et al.* 2004. Inverse correlation between maternal weight and second trimester circulating cell-free fetal DNA levels. *Obstet. Gynecol.* **104**: 545–550.
15. PAN, P.D. *et al.* 2005. Cell-free fetal DNA levels in pregnancies conceived by IVF. *Hum. Reprod.* **20**: 3152–3156.
16. WATAGANARA, T. *et al.* 2005. Placental volume, as measured by 3-dimensional sonography and levels of maternal plasma cell-free fetal DNA. *Am. J. Obstet. Gynecol.* **193**: 496–500.
17. POON, L.L.M. *et al.* 2002. Differential DNA methylation between fetus and mother as a strategy for detecting fetal DNA in maternal plasma. *Clin. Chem.* **48**: 35–41.
18. CHIM, S.S.C. *et al.* 2005. Detection of the placental epigenetic signature of the maspin gene in maternal plasma. *Proc. Natl. Acad. Sci. USA* **102**: 14753–14758.
19. LI, Y. *et al.* 2004. Size separation of circulatory DNA in maternal plasma permits ready detection of fetal DNA polymorphisms. *Clin. Chemistry* **50**: 1002–1011.
20. LI, Y. *et al.* 2005. Detection of paternally inherited fetal point mutations for beta-thalassemia using size-fractionated cell-free DNA in maternal plasma. *JAMA* **293**: 843–849.
21. FINNING, K. *et al.* 2004. A clinical service in the UK to predict fetal Rh (Rhesus) D blood group using free fetal DNA in maternal plasma. *Ann. N.Y. Acad. Sci.* **1022**: 119–123.

22. FINNING, K. *et al.* Prediction of fetal K, c and E blood groups from free fetal DNA in maternal plasma. *Transfus. Med.* 2006. In press.
23. TSUI, N.B.Y. *et al.* 2004. Systematic micro-array based identification of placental mRNA in maternal plasma: towards non-invasive prenatal gene expression profiling. *J. Med. Genet.* **41**: 461–467.
24. WONG, B.C.K. *et al.* 2005. Circulating placental RNA in maternal plasma is associated with a preponderance of 5' mRNA fragments: implications for non-invasive prenatal diagnosis and monitoring. *Clin. Chem.* **51**: 1786–1795.
25. HULTEN, M. 2005. The SAFE network of excellence. *Clin. Chem.* **51**: 7.
26. TABACK, B. & D.S. HOON. 2004. Circulating nucleic acids in plasma and serum: past, present and future. *Curr. Opin. Mol. Ther.* **6**: 273–278.
27. MORI, T. *et al.* 2005. Predictive utility of circulating methylated DNA in serum of melanoma patients. *J. Clin. Oncol.* **23**: 9351–9358.
28. PAN, H. *et al.* 2005. Loss of heterozygosity patterns provide fingerprints for genetic heterogeneity in multistep cancer progression of tobacco smoke-induced non-small cell lung cancer. *Cancer Res.* **65**: 1664–1669.
29. KHAN, S. *et al.* 2004. Genetic abnormalities in plasma DNA of patients with lung cancer and other respiratory diseases. *Int. J. Cancer* **110**: 891–895.
30. HOLDENRIEDER, S. *et al.* 2005. Circulating nucleosomes and cytokeratin 19-fragments in patients with colorectal cancer during chemotherapy. *Anticancer Res.* **25**: 1795–1801.
31. HAMAOU, K. *et al.* 2004. Concentration of circulating rhodopsin mRNA in diabetic retinopathy. *Clin. Chem.* **50**: 2152–2155.
32. LAM, N.Y. *et al.* 2003. Time course of early and late changes in plasma DNA in trauma patients. *Clin. Chem.* **49**: 1286–1291.
33. LAM, N.Y. *et al.* 2005. Plasma DNA as a prognostic marker for stroke patients with negative neuroimaging within the first 24h of symptom onset. *Resuscitation* Nov 30 [Epub ahead of print].
34. KOTSCH, K. *et al.* 2004. Enhanced granulysin mRNA expression in urinary sediment in early and delayed acute renal allograft rejection. *Transplantation* **77**: 1866–1875.
35. MUTHUKUMAR, T. *et al.* 2005. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N. Engl. J. Med.* **353**: 2342–2351.
36. DING, C. & C.R. CANTOR. 2003. A high-throughput gene expression analysis technique using competitive PCR and matrix-assisted laser desorption ionization time-of-flight MS. *Proc. Natl. Acad. Sci. USA* **100**: 3059–3064.
37. DING, C. *et al.* 2004. MS analysis of single-nucleotide differences in circulating nucleic acids: Application to non-invasive prenatal diagnosis. *Proc. Natl. Acad. Sci. USA* **101**: 10762–10767.
38. CIRIGLIANO, V. *et al.* 2004. Rapid prenatal diagnosis of common chromosome aneuploidies by QF-PCR. Assessment on 18,000 consecutive clinical samples. *Mol. Hum. Reprod.* **10**: 839–846.