Circulating Nucleic Acids in Plasma and Serum

Peter B. Gahan
Editor

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Part I
Current Developments

Chapter 1
Current Developments in Circulating Nucleic Acids in Plasma and Serum

Peter B. Gahan

Abstract DNA and RNA fractions have been isolated from the whole blood, serum, plasma, the surface of blood cells, urine, saliva and spinal fluid from both healthy individuals and patients. The ability to isolate, quantify, and analyze these molecules has led to the identification of specific nucleic acid fragments related to a variety of clinical disorders thereby permitting their early diagnosis and prognosis. This chapter summarizes the work reported in this volume.

Keywords Circulating nucleic acids · Fetal medicine · Oncology · New technology · Biology of CNAPS

Introduction

The current volume concerns the meeting of the sixth international conference on circulating nucleic acids in plasma and serum (CNAPS) held in Hong Kong on 9-11 November 2009. The aim of the meeting was to bring together clinicians and scientists working in this field to present their latest findings on the basic biology, methodology and clinical applications of circulating nucleic acids in blood, urine, cerebro-spinal fluid and saliva.

Since the first publication by Mendel and Métais (1948) reporting the circulation of DNA in blood and its increase in amount in cancer patients, studies have evolved from just considering the amounts of DNA circulating in CNAPS during cancer and other clinical situations (Leon et al. 1977; Koeffler et al. 1973; Tan et al. 1966; Stroun et al. 1989) to more recent developments in the use of DNA, nucleosomes, mRNA and micro RNAs as both early markers and prognostic tools. Most work has concentrated upon the role of isolated DNA fractions and

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nucleosomes and to a lesser degree, mRNA. However, it is clear that the nucleic acids present in blood are derived from a number of sources including the breakdown of cells in the blood - both blood cells and circulating cancer cells, cell-surface bound DNA, the presence of bacteria and viruses, tissue necrosis, cell and tissue apoptosis, release of a newly synthesized DNA/RNA lipo-protein complex (the virtosome), exosomes, transposons and retrotransposons (Gahan and Stroun 2010). This medley of sources offers range of choice in the nucleic acid fraction to be assessed with respect to a particular disorder. In addition, the development of approaches involving the exploitation of epigenetic events such as methylation and hypermethylation, histone modifications in circulating nucleosomes, RNA-single nucleotide polymorphism, epigenetic allelic ratios and epigenetic-genetic chromosome dosage has offered more sensitive diagnostic methods that are applicable in the clinical environment. The following comments will highlight advances presented at the symposium and will raise questions as to the future developments needed.

Nucleic Acids in Oncology – Diagnosis and Prognosis and Metastases

Diagnosis and Prognosis

Two aspects of CNAPS in oncology have provided areas of development, namely, the clinical application in early diagnosis and prognosis and the other, a better understanding of the origins of metastases.

Nucleosomes have provided the basis for a number of studies in both early diagnosis and prognosis in cancer. Thus, trans-arterial chemo-embollization, the new loco-regional anticancer treatment option for advanced hepatocellular carcinoma patients, has been assessed in terms of serum nucleosome levels by Kohles et al. Although an initial decline was found in nucleosome levels shortly after treatment, by 24 hours there was a marked increase possibly due to the release of nucleosomes from the increased number of necrotic cells. Hence, this may provide a means of estimating the efficiency of the therapy. Likewise for the studies by Fehr et al. on nucleosome levels in patients after treatment by selective internal radiation therapy. This is a loco-regional anticancer treatment option for advanced cancer patients with liver metastases or liver cancer employing Yttrium-90 labelled microspheres.

A similar prognostic value might be available through the results of a preliminary investigation of nucleosome levels in both blood and cerebro-spinal fluid from patients with glioblastoma by Holdenreider et al. They showed that patients developing oedema after operation had a substantial increase in the nucleosome levels in the cerebo-spinal fluid. Additional studies may show this to be a useful marker of the development of post-operational complications.

As a variation on the nucleosome theme, Delizeger et al. examined a modification of the histone fraction of nucleosomes as a marker for colorectal cancer. Hence,

an analysis of the trimethylation of histone H3 lysine 9 (H3K9me3) and histone H4 lysine 9 (H4K20me3), at the pericentric heterochromatin of the nucleosomes circulating in plasma, exploited the H3K9me3/H4K20me3 ratio for normalizing H3K9me3 concentrations. In this way, it was possible to distinguish patients with colorectal cancer (median 0.8) from the healthy group (median 3) and those with multiple myeloma (median 4.7).

An alternative approach in which allelic imbalance and DNA hypermethylation analyses have been combined, has been exploited by Lessard et al. who demonstrated a significantly improved sensitivity for the method to detect prostate cancer. This approach, using loss of heterozygosity combined with hypermethylation, has proved to be more sensitive than the currently used prostate specific antigen serum levels.

Another approach for the detection of prostate cancer was emplyed by Van der Vaart et al. using parallel tagged sequencing of circulating DNA on the GSFLX sequencer from 454 life sciences. A total of ~3600 unique sequences were analyzed and were seen to be distributed over the human genome with a slightly higher mutation rate being observed for DNA obtained from the cancer patients when compared to the control group. A further characterization of this array of sequences was performed by comparative analysis of chromosome distribution, repeat content and epigenetic characteristics of plasma DNA. Satellite repeats attributed to chromosome 12 were elevated in plasma of prostate cancer patients.

Although the average concentration of circulating DNA, measured as LINE-1 repetitive elements, in plasma was shown to be similar in healthy individuals and non-small cell lung cancer patients, Ponomaryova et al. also found that the concentration of cell-surface-bound circulating DNA was significantly low. This correlated with a poor disease prognosis. The ratio of the β-actin gene to LINE-1 was found to be elevated in the cell-surface-bound DNA of the non-small cell lung cancer patients compared to healthy individuals,. Hence, these results indicate a possible role for β-actin gene and LINE-1 fragments circulating in non-small cell lung cancer patients in both tumour detection and prognosis. Ding et al. also found that the quantification of methylated *RASSF1A* after operation provided a useful prognostic biomarker for predicting the recurrence in non-small cell lung cancer patients after curative-intent surgery.

Epigenetic effects in the shape of promotor methylation rates of three tumour suppressor genes from both plasma DNA and cell-surface-bound DNA from gastric cancer patients were considered by Elistratova et al. Methylated forms of p15, MGMT and hMLH1 genes were detected with high rates at stages II, III and IV of gastric cancer. However, no significant correlation was found between epigenetic and protein markers so indicating their independent development in gastric tumor pathogenesis.

A different approach has been taken by Roth et al. in studies on ovarian cancer patients. The concentrations of four circulating microRNAs (miRNA10b, miRNA34a, miRNA141 and miRNA155) were measured in the serum of 59 patients with ovarian cancer and 29 healthy individuals. The levels of total RNA, miRNA10b, miRNA34a, miRNA141, and miRNA155 in ovarian cancer patients,

were significantly higher than those from the healthy controls. A significant correlation was also recorded of increasing amounts of miRNA34a with lymph node metastases.

Metastases

Amongst the DNAs circulating in cancer patients will be DNAs released from tumour cells either by necrosis or apoptosis or as newly synthesised virtosomes (Adams et al. 1997; Anker et al. 1994; Garcia-Olmo et al. 2010; Stroun et al. 1989). CNAPS DNAs can readily enter cells and in some cases be expressed in a way that modifies the biology of the recipients cell (Adams et al. 1997; Anker and Stroun 1972; Anker et al. 1980, 1994; Bulicheva et al. 2008; Ermakov et al. 2008; Garcia-Olmo et al. 2010; Ottolenghi and Hotchkiss 1960; Skvortsova et al. 2008). Thus, it is not only possible for cancer cells circulating in the blood to result in metastases, but also for the DNA released from tumour cells to do likewise.

One of the most common alterations of tumour related DNA found in CNAPS DNA from cancer patients is its hypermethylation. Thus, methylated fragments of the RAR2 gene from CNAPS have been shown to be taken up by HeLa and human umbilical vein endothelial cells twice as efficiently as unmethylated fragments. Since the methylated RAR 2 gene fragments are more prevalent than the unmethylated fragments in intracellular traffic, they would appear to pose a higher transformation potential (Skvortsova et al. 2008).

It has been shown that the SW 480 cell line, originating from a human colon carcinoma and containing a point mutation of the K-ras gene on both alleles, can be released in the form of the newly synthesised, virtosomal DNA/RNA-lipoprotein complex containing the mutated K-ras gene. Culturing NIH/3T3 cells in the presence of the non-purified SW 480 cell supernatant containing the virtosome complex resulted in the appearance of transformed foci. The presence of a mutated ras gene in the transfected foci of the 3T3 cells was confirmed by hybridization after PCR and by sequencing the PCR product (Anker et al. 1994). In a similar fashion, the virtosomes released from mouse tumour cell lines J774 cells (leukemia) and P497 cells (glial tumour) entered non-stimulated lymphocytes and resulted in their stimulation to synthesize DNA for cell division (Adams et al. 1997). Therefore, it comes as no surprise that Garcia-Olmo et al. (2010) have proposed the Genometastases concept in which the DNA released from tumour cells into the blood moves to other cell sites – possibly stem cells – which are transformed into secondary tumours (Garcia-Olmo et al. 1999). Experimental evidence comes from studies by Garcia-Olmo et al. (2010) in which cultures of NIH-3T3 cells were supplemented with samples of plasma from patients with either K-ras-mutated colorectal tumours or from healthy subjects. This was made by either direct addition of plasma to cultures in standard plates or avoiding plasma-cell contact by placing membranes with 0.4 μm pores between the plasma and the cultured cells to act as a filter and so avoid the involvement of any free host cancer cells. Human gene transfer occurred in most

cultures of NIH-3T3 cells, as shown by the presence of human K-ras sequences, p53 sequences and ß-globin encoding sequences. Furthermore, the NIH-3T3 cells were shown to be oncogenically transformed after being cultured with plasma from colon cancer patients by the development of carcinomas in NOD-SCID mice injected with the transformed NIH-3T3 cells. Cultures with an artificial membrane containing 0.4 μm diameter pores placed between the NIH-3T3 cells and the plasma gave similar results showing that the transforming factor was smaller than 0.4 μm (Garcia-Olmo et al. 2010). The presence of small vesicle-like structures was confirmed by Serrano-Heras et al. through the demonstration of an increased release of DNA-containing vesicles in the bloodstream of tumour bearing, compared to normal, rats. The DNA was shown to contain K-ras sequences and, hence, may be the source of the transforming DNA in the bloodstream. This is strong confirmation to the idea that circulating DNA released from tumour cells can be the direct cause of metastases (Garcia-Olmo et al. 1999).

Nucleic Acids in Foetal Medicine

Pregnant women often opt for prenatal diagnosis to test for foetal chromosomal aneuploidies, the most common aneuploidies including trisomy 21, trisomy 18, trisomy 13 (Savva et al. 2010) and monosomy X in females (Ranke and Saenger 2001). This usually involves the invasive procedures of chorionic villus sampling and amniocentesis in order to obtain foetal genetic material for analyses, such procedures, at times, resulting in the loss of the foetus (Tabor et al. 1986).

The discovery that cell-free foetal DNA contributes a mean of 3–6% of the total maternal plasma DNA (Lo et al. 1998b). has permitted the development of some methods that have already been translated into clinical use e.g. the non-invasive determination of fetal rhesus D status (Lo et al. 1998a; Daniels et al. 2009) and the exclusion of sex-linked disorders (Costa et al. 2002). The early approaches focussed on the detection of foetal-specific RNA/DNA molecules for chromosome dosage determination involving RNA-single nucleotide polymorphism (SNP), epigenetic allelic ratios and epigenetic-genetic chromosome dosage. Tong et al. presented a highly sensitive polymorphism-independent approach using a very precise digital polymerase chain reaction platform together with a single molecule counting technology and a parallel sequencing platform for the direct detection of foetal chromosomal aneuploidies from maternal plasma.

An alternative analyses has been considered by Hultén et al. in which methylated DNA immunoprecipitation in combination with high resolution oligonucleotide microarray analysis has permitted the identification of chromosomal DNA methylation patterns using a high-throughput approach. The methylation patterns of chromosomes 13, 18, 21 and the sex chromosomes in female peripheral blood, CVS and placental DNA will form the basis of non/minimally-invasive prenatal analysis. Morozkin et al. have employed fluorescent *in situ* hybridization to examine extracellular DNA versus genomic or apoptotic DNA from culture medium and bound to the cell surface of human primary endotheliocytes, human primary fibroblasts

and HeLa cells. An over-representation was found for chromosome 9 fragments and the regions of the short arms of chromosomes 13, 14, 15, 21, 22 in DNA isolated from the culture medium of primary fibroblasts. These finding offer DNA targets for diagnostic purposes.

While the above approaches are important in identifying some chromosomal abnormalities, foetal sex and Rhesus factors in the first trimester, only a relatively small fraction of foetuses are affected with trisomy 21. There are many infants and children with a variety of developmental disorders that are not due to aneuploidy and who could benefit from a real-time genomic approach to better understand foetal development and to identify key genes involved in the pathogenesis of disorders such as a means of targeting for therapy. Working with neonatal mRNA rather than DNA, Maron and Bianchi in a "fluid agnostic" approach, have concentrated on mRNA fractions from maternal and neonatal whole blood, amniotic fluid, and neonatal saliva as potential sources of genomic information that could assist an understanding of foetal development, pathology, and diagnosis. Working with mRNA will give a better chance to study differentially regulated genes and so expand the range of developmental and pathological targets.

Other Clinical Exploitation of CNAPS

The level of circulating DNA has been shown to increase in patients presenting with injury, the concentration relating to the severity of the injury (Lan et al. 2003). DNA measurement on admission could be used to predict the outcome in terms of organ failure, acute lung injury, acute respiratory syndrome and death. Similarly, ß-globulin DNA concentration was found to be higher in patients presenting with stroke and could be used as a predictor of death (Rainer and Lam 2006) as were nucleosomes (Geiger et al. 2007). A new duplex real-time PCR assay with internal control developed by Chen et al. was used by them to study circulating plasma DNA levels in trauma patients from the Wenchuan, China earthquake in 2009. During the early stage of injury, the median plasma DNA level of patients was more than five times that of the healthy controls and a statistically significant difference of plasma DNA concentration between patients with and without organ injury was determined.

Cerebrovascular accidents are also characterized by the increase in low molecular weight DNA concentration in the course of 3 days after acuity with a maximum after 3 hours in the case of hemorrhage and after 24 hours in the case of ischemia. Recent analysis by Vasilyeva et al. of such low molecular weight DNA from the spinal fluid from patients with severe cerebral vascular circulatory problems showed a sharp increase within 3 h from the start of the attack, similar to that seen with the DNA fraction from blood. However, since the spinal fluid contains no blood cells during the first 24 h after the attack, the DNA is likely to have the brain lesion as its source.

Horinek et al. have found that plasma DNA levels increase sharply in patients undergoing dialysis and although the levels drop subsequently, they do not return to the control values. The increased DNA levels could be due to apoptosis as shown by over-expression of the pro-apoptotic genes *BAX* and CASP*8*.

The Biology of CNAPS

As has already been mentioned, there are a variety of sources of CNAPS and there are many questions relating to the biology of these nucleic acid fractions to be answered. In some instances CNAPS have been demonstrated to be actively released from cells, readily taken up by other cell populations and biologically active (reviewed Gahan and Stroun 2010). The mechanisms controlling the production and release of both the DNA and RNA fractions, the mechanisms of release and uptake and the way in which they can modify the recipient cell's biology are still to be clarified.

Methylated DNA enters cells more easily than non-methylated DNA as shown by the uptake of methylated fragments of RAR2 gene into HeLa and human umbilical vein endothelial cells being twice as efficient as that of unmethylated fragments. A common alteration of tumour related DNA found in CNAPS concerns the hypermethylation of DNA from cancer patients. Since the methylated RAR 2 gene fragments are more prevalent than the unmethylated fragments in intracellular traffic, they would appear to pose a higher transformation potential (Skvortsova et al. 2008). Skortsova et al. have gone on to show that when human CNAPS is injected into mice methylated DNA was degraded less quickly than the unmethylated form. In addition, a quantitative study of RARbeta2 gene methylation in cell-free DNA and genomic DNA of primary and transformed cells showed an over-representation of methylated DNA sequences in the circulating DNA of primary cells.

From the results of Korabecna et al., it would appear that plasma DNAase II makes only a minor contribution to the degradation of circulating DNA.

A number of studies have reinforced the concept that the circulating nucleic acids can enter host cells and modify the biology of those cells. Thus, Malinovskaya et al. have shown that CpG-enriched rDNA accumulating in human cfDNA significantly stimulates gene transcription in mesenchymal stem cells by activating TLR9 and MyD88-dependent signaling pathways and inhibiting differentiation of mesenchymal stem cells into adipocytes. Inhibition of Poly(I:C)-activated IL-6 and IL-8 Production in Human Primary Endothelial Cells and Fibroblasts was also demonstrated by Cherepanova et al.

The accumulating CpG-rich ribosomal repeat was demonstrated by Efremova et al. to influence brain cell function in pathology and injury being accompanied by intensive DNA liberation from cells as the result of apoptosis or necrosis. pBRTRRR significantly up-regulated iNOS gene expression, being more effective in low concentrations as was the case for iNOS gene expression. Similarly, Alexseeva et al. also showed that cell free DNA could influence the elaboration of NO and ROS depending upon the sample concentration and the content of CG-DNA marker with cell free DNA isolated from blood from patients with cardiovascular diseases influencing ROS synthesis more efficiently than did cell free DNA from healthy donors.

Ermakov et al. have previously shown that low-dose ionizing radiation induced in human G_0-lymphocytes the development of an adaptive response that was accompanied by transposition of homologous-chromosomes loci within the cell nucleus

and activation of the chromosomal nucleolar-forming regions. Such reactions were transmitted to unirradiated lymphocytes via the bystander-effect mechanism (Ermakov et al. 2008). Similar results have been obtained with monolayer mesenchymal stem cell cultures after the development of the radiation induced bystander effect.

Few studies have been made on the effects of circulating RNA to enter cells though RNA has previously been shown to be capable of transforming cells (Skog et al. 2008). Semenov et al have developed a number of analogues of both single- and double-stranded RNAs that have readily entered cells and produced a variety of biological effects in the recipient cells.

New Technology

Although the use of massively parallel sequencing (Rogers and Ventner 2005) has facilitated the development of the analyses of CNAPS such as for diagnosis of trisomy 21 (Chiu et al. 2010), the inclusion of CNAPS as a major player in predictive and preventive medicine will depend upon the reliability of the way that the withdrawn blood is handled prior to nucleic acid extraction and the mechanism employed for the extraction of the nucleic acids as well as the development of rigorous and repeatable techniques linking a particular nucleic acid fragment to a specific clinical disorder.

A way forward designed by Krishnan and Heller allows the nucleic acids to be directly removed from whole blood, even immediately after withdrawal. Using a microarray dielectrophoretic system, high molecular weight DNA can be both detected and rapidly isolated directly from whole blood. Levels of < 260 ng per ml DNA are detectable. The method can also be applied for the isolation of nanoparticles at $< 9.5 \times 10^9$ particles per ml.

Given the variability between available commercial methods for the extraction of nucleic acids from plasma and serum, Fleischhacker et al. have made a comparison of three kits in an effort to establish which kit yields the most DNA isolated versus the immunological quantification of circulating nucleosomes using the Cell Death Detection ELISA plus. The study was performed simultaneously in two separate laboratories. Comparable results were obtained with large differences being recorded between the different procedures and with the MagNA-Pure isolation system giving the highest DNA yield.

The isolation of nucleic acids from plasma, serum and urine has been improved by the development of a new QIAamp® Circulating Nucleic Acid Kit by Horlitz et al. This large volume kit yields 7–9 times as much as that derived with the the QIAamp Blood Mini as well as offering improved recovery of short DNA fragments. It would appear that the QIAamp Circulating Nucleic Acid Kit can serve as a sample preparation solution for processing up to 5 ml cell-free body fluid and can extract and concentrate circulating nucleic acids, including microRNA, and viral nucleic acids up to 250-fold.

One of the newer developments in CNAPS has been the introduction of microRNAs as early markers for diagnosis and prognosis. One area in which this has been applied concerns the study of Alzheimer disease by van Harten et al. Using the Megaplex protocol with Taqman Array MicroRNA cards on small RNA isolated with the MirVana Paris kit it was possible to isolate all 667 currently known microRNAs from the spinal fluid of Alzheimer patients.

Conclusions

There is still a long way to go before CNAPS will become fully integrated into predictive and preventive medicine. However, a strong beginning has been established in both the technology available and the identification of the relevant nucleic acid fragments linked to specific clinical disorders. The trialling of foetal diagnostic methods in national health programmes in some countries is good evidence for this. Nevertheless, it is clear that quality assured methodologies will be needed for three important areas, namely, whole blood handling prior to nucleic acid extraction, the mechanisms employed for the nucleic acid extraction and the development of rigorous and repeatable techniques linking a particular nucleic acid fragment to a specific clinical disorder for either prediction or prognosis.

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