10. CIRCULATING NUCLEIC ACIDS AS DIAGNOSTIC TOOL

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10.1 Introduction

Circulating nucleic acids are present in small amounts in the plasma of healthy individuals. However the discovery of circulating nucleic acids has long been explored for the non-invasive diagnosis of a variety of clinical conditions. The first studies concerning the detection of circulating DNA were investigated for finding various forms of cancer. Metastasis and recurrence in certain tumour types have been associated with the presence of high levels of cancer-derived DNA in circulation. The detection of fetal DNA in maternal plasma in pregnant women is useful in detecting and monitoring fetal diseases and pregnancy-associated complications. Similarly, levels of circulating DNA in acute medical emergencies including trauma and stroke, have been reported to be increased and have been explored as indicators of clinical severity. In the last few years, other than circulating DNA, much attention and effort has been put into the study of circulating RNA, starting 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 fetal RNA detection looks to be a promising approach for the development of gender- and polymorphism-independent fetal markers for prenatal diagnosis and monitoring complications during pregnancy. This development also opens up the possibility of non-invasive prenatal gene expression profiling by maternal blood analysis.

10.2 Noninvasive prenatal diagnosis

The discovery of cell-free fetal DNA in maternal plasma by Dennis Lo in 1997, outlined new scenarios for non invasive prenatal diagnosis. The quantitative analysis of the free fetal DNA showed that this can be made up of as much as 6.2% of the total DNA present in the maternal plasma (1). A deep and extensive search for non-invasive techniques of fetal DNA sampling has been carried out to substitute invasive prenatal diagnosis that carry a significant risk of miscarriage.

Fetal DNA release into maternal plasma has been shown to be a very early physiological phenomenon increasing progressively throughout pregnancy (2-3). Circulating fetal DNA molecules (SRY gene) have been detected in maternal plasma in the first trimester (starting from the 5^{th} gestational week) onwards with an accuracy approximately of the 100% (2-3). This approach has been used for the prenatal investigation of sex-linked diseases and fetal RhD status condition in which the fetus presented a gene absent in the mother .

A majority of research groups use sequences of chromosome Y in male embryos as a marker of fetal DNA and standardization of the assays, due to the fact that a woman (46,XX) does not possess this chromosome in her genome. Sexing analysis is also important, mainly for diseases with a recessive X-linked pattern of inheritance, with female being normal or being carriers of the mutation, but healthy, while male are normal or affected by the disease. An

application of fetal sexing is Congenital Adrenal Hyperplasia (CAH) an autosomal recessive genetic disease which carries a defect in 21- hydroxylase deficiency. Homozygous girls for this pathology are born with masculinization of the external genitalia and often require surgical operations as opposed to affected boys whom present normal external genitalia. Prenatal treatment of CAH with dexamethasone to prevent genital ambiguity has been successfully used (4). However, to minimize the side effects, the interruption of therapy has been indicated in the case of affected or normal male embryos and normal female embryos. For this reason, fetal sexing is necessary during pregnancy and is usually carried out by invasive methods. Noninvasive fetal sexing based on free fetal DNA in maternal plasma would bring the additional advantage of early discontinuation of medication in the case of male embryos (5).

Moreover, many genetic diseases are caused by mutations that result in subtle differences between the sequences of maternal and fetal DNA, such as achondroplasia (6), â and ß thalassemia (7- 9).

Rh alloimmunization is a crucial problem in medical and obstetrical clinical practice, potentially leading to hemolytic disease in the newborn. For pregnant negative Rh women (15% of the population), a positive Rh embryo involves a 16% risk of sensitization to the Rh antigen. Diagnostic procedures and invasive therapy may be necessary to reduce perinatal mortality of positive Rh embryos (10). Thus, the early detection of fetal RhD status through fetal DNA in the plasma of negative Rh mothers is of great importance in defining the need for interventions, with known risks of gestational loss, or of gestational immunoprophylaxis.

10.3 Preeclampsia/Intrauterine Growth Restriction (IUGR)

Preeclampsia is a hypertensive disorder affecting approximately 5% of pregnancies and is still one of the main causes of mortality of both the fetus and the mother. Typical symptoms are maternal hypertension and proteinuria, which usually develop in the late second or third trimester of pregnancy (11).

Increased fetal DNA release can be a marker of pathological conditions affecting both the fetus and the placenta (12-19). Abnormal placentation has often been found to be involved in the pathogenesis of intrauterine growth restriction (IUGR) and preeclampsia, which can occur either isolated or in combination. IUGR is defined as the presence of an ultrasonographically estimated fetal weight below the fifth percentile confirmed post-natally, in the absence of chromosomal and structural abnormalities. Preeclampsia and IUGR have been linked to abnormalities in trophoblast invasion into the placental bed. During normal pregnancy, trophoblastic invasion of uterine spiral arteries takes place reducing the vascular resistance and allowing adequate fetoplacental blood supply. In IUGR and preeclampsia this adaptive phenomenon is often insufficient, resulting in a diminished infiltration and modification of the spiral arteries, which lead to the maintenance of a high-resistance uterine circulation (20-21). Several studies have addressed the issue of quantifying fetal DNA in maternal plasma in pregnancies complicated by preeclampsia, and there is a general agreement of up to fivefold increased fetal DNA levels in the presence of this pathology (19, 22).

Since IUGR is mostly caused by impaired placental perfusion, similar to what is found in preeclampsia, it might be also associated with high levels of fetal DNA in maternal circulation.

The increase in the rates of circulating fetal and maternal DNA would correspond to the degree of severity of the illness and, therefore, the level of fetal DNA may serve as a marker of the prognosis and severity of the clinical picture (23-24).

Although most researchers use the Y-chromosome in this specific application, other non-gender markers have been studied, including epigenetic markers, to improve the number of pregnant women that could be submitted to quantitative investigation (25).

Recently, fetal RNA has also been found in maternal plasma. Such fetal RNA has been shown to originate from the placenta and to be remarkably stable. The use of microarray-based approaches has made it feasible to rapidly generate new circulating RNA markers. It is hoped that further developments in this field will make the routine and widespread practice of noninvasive nucleic acid–based prenatal diagnosis for common pregnancy-associated disorders feasible in the near future.

10.4 Cancer

Cancer is a common malignant disease in industrialised countries. Early diagnosis of tumours and accurate identification of haematogenic metastases can improve the success of treatment (26–29). Therefore, the detection of single tumour cell released in the blood in early stages could help physicians choose the most advantageous therapy for patients. The presence of small amounts of cell free tumor DNA (cfDNA) circulating in the plasma or serum of cancer patients was first demonstrated 30 years ago and provides another possibility of examining tumour derived genetic material in the circulation and to detect haematogenic spread of tumour cell DNA (30). Qualitative alterations in circulating DNA, such as microsatellite alterations (31), oncogene mutations (32), mitochondrial DNA, tumour-specific methylated DNA (33) and viral DNA (34), have been found in patients with different types of cancer. Quantitative alterations of circulating cfDNA have also been observed in several tumours, such as prostate cancer (35), lung cancer (36), pancreatic cancer (37), leukaemia and lymphoma (38). High levels of circulating cfDNA were correlated with tumour metastasis, response to therapy and recurrence (36-39). Therefore, tumour-derived circulating nucleic acids in the plasma or serum of cancer patients were introduced as a tool for detection and surveillance of cancers (40).The proportion of patients with altered cfDNA varies with the pathology and the nature of the marker. However, several studies have reported the presence of altered cfDNA in over 50% of cancer patients (41), suggesting that this marker may be common and amenable for a variety of clinical and epidemiological studies. Because the mechanisms and timing of cfDNA release in the blood stream are poorly understood, only few studies have addressed the use of cfDNA for early cancer detection or as a biomarker for mutagenesis and tumourigenesis in molecular epidemiology. (41). In some circumstances, cfDNA alterations are detectable ahead of cancer diagnosis, raising the possibility of exploiting them as biomarkers for monitoring cancer occurrence.

10.5 Trauma

The mechanisms by which cell-free DNA is freed into the circulation of human subjects are unknown; one possibility is that DNA is released following cell death (42-43).

It is also possible that direct damage or hemodynamic compromise of the organ systems responsible for circulating DNA clearance may also lead to increased plasma DNA. Candidate

organ systems include the liver, spleen, and kidneys, which may have a role in both liberating and clearing circulating DNA.

Along this line of reasoning, Lo's group hypothesized that DNA may be liberated from body tissues into the plasma after trauma and that plasma DNA may be a potentially useful prognostic tool (44).

In this study Lo shows that circulating plasma DNA in the peripheral blood of trauma patients increases early after injury and that these increases are related to the development of posttraumatic complications, suggesting that plasma DNA may be a potentially useful marker for monitoring patients after trauma.

10.6 Stroke

Stroke ranked as the second leading cause of all deaths worldwide in 1990, accounting for 4.4 million victims (45), and is also currently the leading cause of brain injury in adults (46). Preventive strategies have led to a decrease in the rate of stroke attacks and deaths. Increased concentrations of several neurobiochemical protein markers have been detected in the peripheral blood of patients with stroke, but at present there is no simple and accurate blood test that may be used to determine the severity of a stroke or to predict mortality and morbidity in stroke patients on arrival in emergency wards in clinical practice.

In Lo's paper, the authors concluded that plasma DNA concentrations correlate with stroke severity and may be used to predict mortality and morbidity in the emergency room (47).

As both hemorrhagic and ischemic strokes (48) involve cell death and disruption of the blood–brain barrier, they hypothesized that DNA would be liberated into the plasma early after the onset of stroke and that it might be useful for assessing disease severity and for predicting mortality.

They have also shown that plasma DNA measurements may be useful for early risk stratification and for predicting in-hospital and 6-month disability and mortality. The greatest differences in plasma DNA concentrations between patients with good and poor outcomes occurred within 3 h of the onset of symptoms.

The mechanisms by which circulating cell-free DNA increases after stroke require further study but are likely to be a result of increased liberation from damaged cells. Strokes involve a complicated cascade of events involving cerebral ischemia, altered cerebral blood flow, inflammation, the production of reactive oxygen radicals, neuronal necrosis and apoptosis, and neurologic dysfunction (49-53). DNA may be liberated from cells undergoing apoptosis or necrosis.

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