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Review article

Cell-free nucleic acids in urine as potential biomarkers of kidney disease

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ABSTRACT

Kidney and uropoetic system diseases represent a major social, economic and health burden. This is mainly because early diagnosis of kidney dysfunction is currently unavailable, since the current markers are often reliably increased only after advanced progression of the renal diseases. Recently, circulating nucleosomes, DNA and numerous forms of RNA have been detected in human biological fluids, such as plasma, urine, saliva, and breast milk. Although their biological functions remain mostly unknown, they are attractive as potential biomarkers of various diseases. In urine, many of the circulating nucleic acids originate from the cells of the kidney and the urinary tract making these non-invasive and easily obtained new biomarkers in the nephrology or urology. This review focuses on cell free nucleic acids in urine and its potential in human studies. Although, there are some technical and biological limitations, the urinary circulating nucleic acids hold a great potential as new biomarkers of renal diseases.

Introduction

Nucleic acids that are not contained within cells are commonly referred to as circulating nucleic acids (CNA). They were first described in 1928, when they were observed to transmit pathogenicity from heat-killed to viable bacteria (Griffith, 1928). The presence of CNA in the circulation was first reported in 1948 by Mantel and Metais in Comptes Rendus de l'Académie des Sciences, Paris. The discovery was not taken seriously until high concentrations of CNA were detected in the blood of patients with systemic lupus erythematosus in 1966 (Tan et al., 1966).

CNA was already isolated from almost all biological fluids, including plasma, serum, saliva, urine, milk and bronchial lavage (O'Driscoll, 2007). It is generally accepted that CNA circulates in measurable amounts in both healthy individuals, and in patients with various diseases. Leon et al. (1977) detected significantly higher concentrations of CNA in the serum of patients with different types of cancer when compared to healthy individuals. They also showed that the concentrations of CNA decreased after patients were successfully cured (Leon et al., 1977; Mittra et al., 2012). This pointed at tumour-derived DNA to be one of the major sources of CNA in cancer patients (Chan et al., 2003). But the existence of CNA was not only found to be interesting for the field of oncology. Lo et al. (1997) were the first to demonstrate that the fetal DNA is circulating in maternal plasma and serum during pregnancy. Also, a fraction of CNA in plasma of transplanted patients has been shown to be of donor origin. This form of DNA chimerism proved to be useful in the monitoring of allograft rejection (Lo et al., 1998). DNA from infectious agents was also found to be a source of CNA. DNA from *Plasmodium* sp. has been detected in the plasma of patients with malaria (Gal et al., 2001). Inflammation related to overtraining of professional athletes was

Abbreviations: CNA, circulating nucleic acids; SAP, serum amyloid P component; EVs, extracellular vesicles; MVB, multivesicular body; mRNA, messenger RNA; miRNA, micro RNA; lncRNA, long non coding RNA; mtDNA, mitochondrial DNA; DAMPs, damage-associated molecular patterns; NETs, neutrophil extracellular traps; AKI, acute kidney injury; scaRNAs, small Cajal body-specific RNAs; snoRNAs, small nucleolar RNAs; snRNAs, small nuclear RNAs; piRNAs, Piwi-interacting RNAs; COX3, cyclooxygenase 3; ND1, nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit-1; eGFR, estimated glomerular filtration; NGAL, neutrophil gelatinase-associated lipocalin; B7-1, type I membrane glycoprotein; RCC, renal cell carcinoma; B-FABP, brain type fatty acid binding protein; UCA1, urothelial carcinoma associated 1.

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also linked with increased concentrations of plasma CNA (Fatouros et al., 2006). A direct relationship between acute traumatic tissue injury and plasma CNA concentration has been further established. Acute stroke followed by neuron damage can release DNA into the peripheral blood. It has been reported that plasma DNA concentration strongly correlates with the severity of stroke. This finding can be useful in risk stratification of acute stroke patients (Lo et al., 2000; Rainer et al., 2003). Increased plasma CNA, released from apoptotic/necrotic adipocytes, has also been reported in obesity. It has been hypothesized that this CNA released from adipocytes has a leading role in the development of chronic adipose tissue inflammation and insulin resistance in obesity (Nishimoto et al., 2016). As can be seen from the above-mentioned examples, CNA can provide an effective tool for diagnostics of many pathological states. In this review, we provide an overview of extracellular nucleic acids, their origins, and structure with the emphasis on urinary CNA and early diagnostics of renal diseases.

The origin of circulating nucleic acids

There are various origins and forms of CNA. Circulating nucleosomes, naked DNA, several types of RNA, and mitochondrial DNA have all been detected in plasma, serum and other biological fluids (Peters and Pretorius, 2011). Both passive and active releases of CNA have been described.

Two main sources of these CNA have been proved: the first is a "passive" way, in which CNA as fragments of DNA are released during apoptosis and necrosis. The other, "active" way assumes an active metabolic secretion of DNA from live cells into the extracellular space and biological fluids (Gahan et al., 2008). Both theories had supporters in the field of CNA research, and at this point, it can be stated that both seem to be correct.

Between 10^{11} and 10^{12} cells divide daily in the human body and the same number goes through apoptosis, to maintain homeostasis. They leave behind 1–10 g of DNA debris per day, and some of this fragmented DNA and nucleosomes may escape cleavage mechanisms. During necrosis, intact DNA can escape fragmentation, as necrosis does not involve intranucleosomal degradation. However, it has been shown that the necrotic pathway is not a major contributor to CNA release (Jahr et al., 2001; van der Vaart and Pretorius, 2008). Terminal differentiation is also a passive source of CNA, as the final stages of the differentiation of erythrocytes, keratinocytes, and lens crystalline cells. In these, the extrusion of nuclear material from the cell occurs (Appleby and Modak, 1977; Scher and Friend, 1978; Terada et al., 1978; Weil et al., 1999).

The active release of DNA by living cells *in vitro* was firstly described by Anker et al. (1975). It has also been shown that viral DNA circulates in the plasma of patients with cancers associated with the viral infections. In nasopharyngeal carcinoma patients, DNA from Epstein–Barr virus can be detected in 96% of cases, and human papilloma virus DNA is present in 50% of patients with cervical cancer. Thus, viral DNA represents an additional source of CNA (Lo et al., 1999a,1999b; Raptis and Menard, 1980; Yang et al., 2004).

Under normal circumstances, pure forms of nucleic acids circulating freely in plasma are degraded because of plasma and serum endonucleases. CNA circulate in complexes with proteins and lipids that protect them from degradation (Fleischhacker et al., 2011). In plasma, several enzymes are actively degrading DNA, such as deoxyribonuclease I, deoxyribonuclease II, phosphodiesterase I and DNA hydrolysing autoantibodies. The most active is deoxyribonuclease I in plasma. (Frittitta et al., 1999; Nadano et al., 1993; Shuster et al., 1992; Yasuda et al., 1992). The fact that DNase has been shown to be inhibited in tumours can partially explain why patients with tumours have higher concentrations of plasma CNA. In these cases, it is possible for free DNA to circulate with lesser protection (Cooper et al., 1950; Tamkovich et al., 2006). DNA segments, which circulate in the form of nucleosomes (DNA wrapped around histone protein cores), are partially protected against degradation. However, the half-life of the nucleosomes that are injected intravenously is the same as the half-life of fetal DNA in maternal plasma (Gauthier et al., 1996; Lo et al., 1999a,1999b).

Another form of protection for DNA is serum amyloid P component (SAP), a 25 kDa pentametric serum protein, which is able to bind to nucleosomes by replacing histone H1. It then proceeds to protect DNA from degradation by nucleases and makes nucleosomes soluble in plasma (Pepys and Butler, 1987).

Apoptotic bodies are another source of CNA in the plasma. Apoptotic bodies contain DNA or RNA, and are formed during apoptosis. DNA is cleaved, and the cell and the nucleus are segmented into small uneven pieces and wrapped in membranes. They can escape phagocytosis by macrophages and appear in the plasma as CNA. It has been shown that SAP can bind apoptotic bodies as well (Bickerstaff et al., 1999; Halicka et al., 2000).

Small intracellularly generated extracellular vesicles (EVs) may contain nucleic acid cargo, which is protected from degradation. These include smaller exosomes ranging from 30 nm to 100 nm in diameter, and larger microvesicles sized between 100 nm and 1000 nm. Exosomes are released by a cell when a late endosome, also called a multivesicular body (MVB), fuses with the plasma membrane and releases its contents. Microvesicles differ from exosomes in biogenesis: they arise by the outward budding and fission of the plasma membrane (Akers et al., 2013). These vesicles play a role in intercellular communication and have been implicated in many processes, such as tumour progression or angiogenesis. Recently, it was shown that their cargo may include a set of nucleic acids. EVs have been found to contain messenger RNA (mRNA), microRNA (miRNA), long non-coding RNA (lncRNA) – and mitochondrial DNA (mtDNA). Exosomal mRNA can be translated in target cells, representing a way of intercellular communication. Exosomes derived from tumour cells have been reported to carry whole genomic dsDNA from the parental tumour cells (Gezer et al., 2014; Guescini et al., 2010; Montecalvo et al., 2012; Thakur et al., 2014; Urbanelli et al., 2015; Valadi et al., 2007).

Tumour associated circulating RNA was first described in the plasma of cancer patients by Kopreski et al. (1999). Since then, mRNA and miRNA were both found circulating in the plasma of healthy subjects and patients. miRNAs are a 19–23 nt long molecules responsible for the post-transcriptional regulation of gene expression. They were first detected in human plasma by Chim et al. (2008). Later, it was found that miRNA specific for placenta was present only in pregnant women and disappeared after delivery (Chim et al., 2008). miRNAs are highly stable in plasma and they can withstand RNase attacks. They occur mostly in lipid or protein complexes, or in vesicles, such as apoptotic bodies, exosomes or microvesicles. They were also found to be associated with ribonucleoprotein complexes and in association with argonaute proteins (Arroyo et al., 2011; Turchinovich et al., 2011; Wang et al., 2010).

mtDNA circulating in the plasma was firstly described by Chiu et al. (2003). Under physiological conditions, mtDNA is present inside the mitochondrial matrix. When a mitochondrial injury occurs, mtDNA fragments, also known as mitochondrial DAMPs (damage-associated molecular patterns) can escape to cytosol and enter systemic circulation (Wenceslau et al., 2014). Patients suffering from age-related neurodegenerative disorders have shown that mtDNAs in human plasma exist as the particle-associated and free state. mtDNA has also been described in exosomes and in relation to neutrophils (Mehra et al., 2007; Tsai et al., 2011; Zachariah et al., 2008). Circulating mtDNA has been reported in relation to various pathologies, e.g. higher

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