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Recovering circulating extracellular or cell-free RNA from bodily fluids

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ABSTRACT

The presence of extracellular circulating or cell-free RNA in biological fluids is becoming a promising diagnostic tool for non invasive and cost effective cancer detection. Extracellular RNA or miRNA as biological marker could be used either for the early detection and diagnosis of the disease or as a marker of recurrence patterns and surveillance. In this review article, we refer to the origin of the circulating extracellular RNA, we summarise the data on the biological fluids (serum/plasma, saliva, urine, cerebrospinal fluid and bronchial lavage fluid) of patients suffering from various types of malignancies reported to contain a substantial amount of circulating extracellular (or cell-free) RNAs and we discuss the appropriate reagents and methodologies needed to be employed in order to obtain RNA material of high quality and integrity for the majority of the experimental methods used in RNA expression analysis. Furthermore, we discuss the advantages and disadvantages of the RT-PCR or microarray methodology which are the methods more often employed in procedures of extracellular RNA analysis.

1. Introduction

Despite the advances in cancer therapeutic approaches, during the last decades, the morbidity and mortality rates in cancer still remain high [1]. The earliest possible diagnosis and treatment is still the best approach to improve survival rates to the disease [2]. The National Cancer Institute estimates that premature deaths, which may have been avoided through screening, range from 3% to 35% [3]. Screening for cancer is usually attempted whenever worrying symptoms arise, having as a result the diagnosis of cancer as a late stage disease [4]. The current methods for diagnosis of the disease are usually invasive (e.g. PAP smear, colonoscopy, etc.) and expensive whereas the existing biological markers are not definitive and lack high sensitivity and specificity [5]. Many researchers therefore work on developing new, sensitive and inexpensive methods for cancer diagnostics [6]. Detection of extracellular or cell-free nucleic acids (DNA or RNA) in plasma, serum and other bodily fluids using the PCR or RT-PCR methods have been suggested as non invasive and cost effective methods for cancer detection [7]. The first report on nucleic acid detection in plasma, was back in 1972 [8] and since then numerous are the reports on detection of cancer specific mutations in circulating DNA or RNA in plasma [9], as well as in other bodily fluids [10–12].

More recently, mRNA biomarkers in serum, plasma and other bodily fluids have been analyzed performing reverse transcription-PCR (RT-PCR)-based detection strategies in patients with cancer [13]. Parallel to the increasing number of such biomarkers in bodily fluids is the growing availability of technologies that enable mass screening programs [14].

Detection of mRNA markers compared to conventional biochemical methods, seems to increase specificity, sensitivity and timeliness of the cancer screening method.

2. Origin of circulating extracellular or cell-free nucleic acids

Extracellular or cell-free nucleic acids (DNA or mRNA) have been isolated from various bodily fluids and used as biological markers for various diseases among which is cancer [15–18]. Several terms are used for these extracellular nucleic acids such as: (1) Circulating nucleic acids, mainly used for DNA and RNA, circulating in plasma or serum (2) Extracellular, or (3) Cell-free nucleic acids, when isolated from other body fluids such as: saliva, urine, cerebrospinal fluid (CSF), bronchoalveolar lavage fluid (BALF) amniotic fluid and other.

Several different mechanisms have been implicated as a potential source for the circulating extracellular or cell-free nucleic acids. One possible mechanism is cell necrosis resulting

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in the presence of high amounts of DNA (and probably RNA) in the plasma of cancer patients with large or advanced tumors [19,20]. Apoptosis has also been considered as another possible source of circulating cell-free nucleic acids. Circulating DNA analyzed by electrophoresis often exhibits a typical ladder of fragmentation similar to that shown by apoptotic cells [21–23]. Apoptotic bodies come from aged or damaged cells. Halicka et al. demonstrated that during apoptosis the nucleic acids, DNA and RNA, are packaged separately into two types of apoptotic bodies, one type contains only RNA and no detectable levels of DNA and the other type contains only DNA and no detectable RNA [24]. Furthermore, it was shown that extracellular, mRNA coding for the enzyme tyrosinase which is associated with apoptotic bodies, is protected, from degradation in human serum [25]. These finding suggests that extracellular RNA, circulating within apoptotic bodies, is less susceptible to ribonucleases' activities [5,25].

Spontaneous, active release of DNA and RNA by tumors in microvesicles is another possibility. Both normal but also cancer cells release microvesicles which are spherical membrane fragments [26] and come from the surface of the cell or the endosomal membranes. Microvesicles are released from viable cells and are usually smaller in size than the apoptotic bodies. Microvesicles are reported to be a normal constituent of human plasma [27] transferring proteins and RNA and contain no fragmented DNA [5,27–41]. In addition, lysis of cancer cells, trauma [5,42] and therapeutic procedures [43–46] might also contribute to circulating nucleic acids.

3. Biological fluids containing circulating extracellular (or cell-free) RNAs

3.1. Plasma/serum

The first reports on increased circulating nucleic acids – both DNA and RNA – in the serum of cancer patients were back in 1977 [47]. Circulating DNA and RNA in the serum of cancer patients were isolated and characterized as tumor-derived and tumor-specific in the late 1980s [23,19]. At first the cancer specific mRNA existing in serum was thought to come from circulating cancer cells [48]. The amount of circulating nucleic acids however cannot be justified by the number of cancer cells existing in serum [9], leading to the hypothesis that tumor specific circulating or cell-free nucleic acids come from the necrosis [19,20] or apoptosis [24,25] of cancer cells or they are even actively released by them in microvesicles [26,27,29].

Circulating RNA has been isolated from the plasma or serum of patients suffering from various types of malignancies such as breast cancer [49–56], lung cancer [57–63], prostate cancer [64–78], thyroid cancer [79–98], hepatocellular carcinoma [5,72,75,99–112], melanoma [113–116], gastric cancer [117], renal cell cancer [118], oesophageal cancer [119,120], rectal carcinoma [121], gynecologic malignancies [122], pancreatic cancer [61], colon cancer [61], bladder cancer [16] and solid tumors [123], and has been used as a biological marker either for the early detection and diagnosis of the disease [64,100], or as a marker of recurrence patterns [81,121], survival predictor [51], follow up and surveillance marker [79,82].

In particular, in breast cancer, measurement of serum metastasin mRNA has been proposed as a screening tool, predicting poor survival and distant metastases [51], whereas measurement of circulating 5T4 mRNA as having the potential to identify patients who could benefit from a 5T4-targeted therapy [53]. Moreover, the measurement of circulating Dkk-1 levels in breast cancer patients could predict bone metastases [56].

In lung cancer, serum hTERT mRNA together with EGFR mRNA was proposed as a good biomarker for diagnosis and clinical stage assessment [58].

In prostate cancer, serum PCA3 mRNA detection [64], HIF-1alpha mRNA quantitative RT-PCR [65], and serum E2F3 mRNA measurement [67], are proposed for the diagnosis of the disease. Furthermore, circulating PSA mRNA was proposed either as a preoperative prognostic marker for organ-confined or locally advanced prostate cancer [71], or as a tool to detect potential surgical failures pre-operatively [77] and monitor patients with metastatic prostate cancer [69].

In thyroid cancer, peripheral thyrotropin hormone receptor (TSHR) mRNA has been proposed for cancer detection in patients with thyroid nodules [87], or in indeterminate fine-needle aspiration [83] or for predicting recurrence [79]. Moreover some authors suggest measurement of serum thyroglobulin mRNA in detecting thyroid cancer [87] or monitoring metastatic disease [89], whereas others do not support this idea [90,91].

In hepatocellular carcinoma, Dong et al. suggested that measuring circulating plasma hepatic TGF-beta1 might prove useful in the diagnosis and prognosis of HBV-induced hepatocellular carcinoma [100]. Wu et al. proposed hepatoma-specific alpha-fetoprotein mRNA (HS-AFP mRNA) and circulating alpha-fetoprotein (AFP)-mRNA [103] and Dong et al., insulin-like growth factor II (IGF-II) mRNA [107] as markers to diagnose hepatocellular carcinoma, monitor metastasis and relapse.

In esophageal squamous cell carcinoma, preoperative squamous cell carcinoma–antigen messenger RNA (SCC–Ag mRNA) levels in the peripheral blood are proposed as predictive factor for recurrence [120].

The detection of circulating fetal RNA (placenta derived) in the maternal circulation has been tested in prenatal diagnostics as a non-invasive procedure compared to amniocentesis, chorionic villus sampling and cordocentesis which are for the moment the routine methods. The first to show that fetal RNA is present in the plasma of pregnant women was Poon in 2000 [124]. Ng et al. in 2003 [125] showed that placenta-derived RNA coding for human placental lactogen and the β-subunit of human chorionic gonadotrophin and corticotrophin-releasing hormone (CRH) [126] are detectable using Real-time PCR in the maternal plasma. Detection of placental circulating RNA in the maternal plasma has also been proposed for the early diagnosis of several pregnancy complications such as preeclampsia [127] and chromosomal aneuploidies [128]. These markers have been shown to be independent of gender and polymorphisms [129]. Moreover, Oudejans et al. in 2003 [130], detected placenta-derived mRNA specific for chromosome 21, whereas Wataganara et al. in 2004 [131] detected fetal-derived gamma-globin mRNA in the maternal plasma. Further more in 2004, Tsui et al. [132] using expression microarrays identified new mRNA species detectable in maternal plasma that can be used in the future as markers in prenatal diagnostics. Establishing these markers in the clinical diagnostic routine is the next step to be taken.

Cell-free DNA was demonstrated to increase in the circulation of patients after acute trauma giving its measurement a possible prognostic ability [133]. On this basis, there was an attempt to measure glyceraldehyde-3-phosphate dehydrogenase mRNA in the plasma of trauma patients which was found significantly increased to that of healthy individuals when plasma was filtered through a 0.22 μm pore size filter [134]. Orlandi et al. attempted to measure circulating mRNA for nephrin in healthy volunteers and renal transplantation patients [135]. They found that mRNA for nephrin reduces significantly with age and transplantation probably due to the reduced renal mass. Circulating mRNA for nephrin was found however higher in females something which must be taken into account when accessing the results of cell-free