Review Article

Circulating nucleic acids in cancer and pregnancy

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ABSTRACT

Circulating nucleic acids are present in the blood of humans and other vertebrates. During the last 10 years researchers actively studied cell-free nucleic acids present in plasma or serum with great expectations of their use as potential biomarkers for cancer and other pathologic conditions. In the present manuscript the main findings related to the principal characteristics of circulating nucleic acids, the hypothesis on their origin and some methodological considerations on sample collection and extraction as well as on some innovative assay methods have been summarized. Recent reports on the importance of circulating nucleic acids in the intercellular exchange of genetic information between eukaryotic cells have been reviewed.

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1. General issues on circulating nucleic acids

Nucleic acids circulating in human blood but also in other body fluids (lymphatic fluid, liquor, ascites, milk, urine, stool and bronchial lavage) can be distinguished as either cell-free (CF) or cell-associated nucleic acids.

Circulating nucleic acids (CNA) have been firstly identified by Mandel and Metais in 1948 [1], but no association with disease was hypothesized. Only 30 years later, in 1977, Leon et al. [2] found CNA in plasma of patients affected by lung cancer.

Different hypotheses were done to explain the origin of CNA. They are supposed to derive from cells dead for necrosis or apoptosis [3], but some authors reported the possibility of an active release from cells [4–8]. The presence of DNA and RNA circulating freely in the blood stream of healthy subjects can be related to activated lymphocytes and to the lysis of other nucleated cells or to their active secretion. In patients affected by neoplastic diseases it is supposed that normal and cancer cells can: (i) detach from the tumor mass and undergo necrosis or apoptosis; and alternatively (ii) actively release nucleic acids in the blood flow.

The quantity of circulating DNA is generally very low in healthy subjects (less than 5 ng/ml of plasma), while it increases (5–10 times) when considering subjects affected by a neoplastic disease [9–12] as well as in some physiologic conditions, such as pregnancy [13,14]. In physiologic conditions as well as in benign pathologies, CF DNA seems to derive almost entirely from apoptosis of circulating cells [15]. CF DNA is double stranded and in the form of nucleoproteic complex. Studies on fetal DNA, reported an half-life of 16.3 min for CF DNA [16] that can be reasonably extended to circulating DNA in general as confirmed by experiments conducted by injecting puri-

fied DNA into the blood stream of animals [17]. Moreover, the pattern of circulating DNA was found to be similar to that of apoptotic cells when analyzed by gel electrophoresis [18,19] and by sequencing reactions [20–23]. Nevertheless also high molecular weight DNA fragments have been retrieved [19] and their presence was associated to the necrosis of tumor cells. Results in this field are often in disagreement. Moreover, all the previous reports regarding the genetic analysis of CF DNA showed that high molecular weight fragments do not contain tumor-specific mutations which on the contrary are frequent in small DNA circulating fragments, probably deriving from phagocitated necrotic cells [24].

Differences in the nature of CF DNA may be an interesting pathological indicator, suggesting that in some pathologies, the measurement of DNA integrity may provide a simple and inexpensive method for cancer detection [3].

As regard to the biological significance of CNA, Garcia-Olmo and co-workers [25,26] suggested that metastases might develop as a result of transfection of susceptible cells in distant target organs with dominant oncogenes that circulate in the plasma and are derived from the primary tumor.

When analyzing the results present in the literature, discordant results can be attributed to several factors, but the source of discrepancies can be the use of plasma or serum as the starting material. Several anticoagulants (EDTA, heparin, and lithium-heparin) have been used and, within the same biological fluids, the variability depends upon the use of different protocol for sample processing (i.e. sample collection and nucleic acid extraction procedure).

In particular, little is known about blood sample processing and especially about the delay in separating the plasma or serum, variation in centrifugation steps and duration and conditions of storage [27]. On the contrary all the studies reported the extraction method used so that the results could be read in the light of the different procedure utilized. Last but not least, every original article

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implies a different technique for detecting the CNA, some being quantitatively oriented, whereas others evaluate only the qualitative aspects. Moreover, even the quantitative real time methods differ in the choice of the template gene and consequently in the assay characteristics.

Thus, the mechanism and the nature of nucleic acids circulating in serum and plasma has not been clarified yet, notwithstanding many articles reported biomarker validations among CNA. Recently, comparison of CNA with genomic DNA by next-generation sequencing [22] suggests that non specific DNA release is not the sole origin for CNA and that apoptotic genomic DNA is the major but not the unique source of CNA in apparently healthy individuals. The authors did not find a circulating DNA pool consisting purely of unspecific apoptotic or necrotic nuclear DNA, failing in evidencing classes of sequences differing from circulating and genomic DNA, apart for the detection of over-represented Alu sequences in serum DNA [22].

The apparent higher content of DNA in serum relatively to plasma can be explained by the clotting process of blood cells that can cause nucleic acid release [28,29]. Concordantly with the above cited papers it was reported that the majority of cell-free DNA in serum samples is generated in vitro by lysis of white blood cells [30]. This is the reason why plasma seems more convenient and adequate for the study of CNA, since its analysis avoids the simultaneous testing of material originally associated to cells, as demonstrated for fetal DNA detection in maternal plasma [31].

2. Updated procedures for sample collection

Relatively to sample collection procedures, it was reported that both a delay in blood processing and storage temperature can influence the amount of DNA extracted from plasma [32]. Anticoagulants do not influence the quantity of the recovered DNA from plasma, but EDTA shows a stabilizing effect on blood during the time between sample draw and processing, both at room temperature and at 4 °C [32,33].

In order to get rid of contaminating DNA derived from cells, both filtration [34] and repeated centrifugations [35–37] at low and high speed were reported, demonstrating that no release of circulating nucleic acid was induced from blood cells even at maximum centrifugation speed. Regarding the stability of CNAs in the frozen samples, some authors showed that plasma can be conserved frozen for years (at least 2 for RNA and 6 for DNA) [38–40] at −70 or −20 °C without affecting CNA concentration, while other authors reported a decay of 30% in DNA from stored plasma [29].

In our lab the following procedure was adopted for plasma sample collection:

(i) Samples are collected in EDTA-containing tubes and they should arrive in lab within 1 h from blood draw.
(ii) They are submitted to a first centrifugation step at 1600*g*, 4 °C for 10 min. Plasma will be recovered.
(iii) A second centrifugation is performed at maximum speed, at 4 °C, for 10 min. Pellets eventually formed in this step will be discarded. Plasma will be split into one-extraction-aliquots of 500 µl each.
(iv) Plasma will be maintained at −80 °C until extracted.

3. Nucleic acid extraction method – CNA extraction

3.1. DNA extraction

The extraction method is an important issue to be addressed in the field of CNA, for which there is no agreement in literature and several protocols have been reported [41].

The isolation of CF DNA from plasma and serum represents a challenge, due to its small quantity and fragmented nature [42].

A recent study [43] compares different commercial kits for DNA isolation (QIAamp DNA Mini Blood Kit by Qiagen, Agencourt Genfind Blood and Serum Genomic DNA Isolation Kit by Agencourt Bioscience Corporation, QIAamp Virus Spin Kit by Qiagen, Invitrogen ChargeSwitch gDNA 1 ml Serum Kit by Invitrogen) reaching the conclusion that the QIAamp Virus Spin Kit gives the best yields from serum and plasma. Other authors [42] reported the superiority of the NucleoSpin Plasma XS Kit by Macherey–Nagel in terms of DNA yield, purity, and retrieval of small DNA fragments with respect to the QIAamp DNA Blood Mini Kit (QIAGEN), which is widely used by investigators in the field of CNA.

In a report on circulating fetal DNA in maternal plasma [44] the NucliSens Magnetic Extraction system (BioMérieux) and the QIAamp DSP Virus Kit (QIAgen) are compared with QIAamp DNA Blood Mini Kit (QIAgen). Both methods were assessed to improve total DNA yield. QIAamp DSP Virus Kit provided the best results for fetal versus total DNA, as it preferentially extracts small size DNA fragments.

In our experience the comparison between the two most widely used kits (DSP and QIAamp) confirmed these findings, as explained in the section dedicated to the DNA integrity index.

3.2. RNA extraction

Also cell-free RNA (CF RNA) suffers from the same pre-analytical problems encountered for CF DNA, with the peculiar difference to show higher stability than that expected on the basis of the characteristics of tissue-extracted RNA.

Thus RNA from plasma survives up to 24 h [45]. Standardized kits specifically designed for viral RNA extraction (i.e. DSP Virus Kit or QIAamp UltraSens Virus Kit, Qiagen, Hilden, Germany) showed appropriate performances for the isolation of CF RNA from human plasma, using silica gel membrane technology. CF RNA was reported to be complexed to other molecules, making it resistant to nucleolytic attacks. Optimized protocols may lead to higher amounts of CF RNA and this finding confirms the importance of pre-analytical factors in CNA isolation [46].

4. Circulating DNA parameters

4.1. DNA quantity

The need for a correct quantification of CF DNA was evident when significant differences between cancer patients and the control population were demonstrated [47,48]. Total plasma DNA concentration is now considered as an unspecific biomarker, but easily identifiable in neoplastic patients as well as in some benign diseases. Initial methods ranged from diphenylamine staining [49], Dipstick Kit [50], counter immuno-electrophoresis [51] spectrophotometric measurement [52], picogreen assay [53] and real-time quantitative PCR (qPCR) [54]. An increased plasma concentration can be observed at an early stage of tumor development [55] as well as during the first trimester of pregnancy [56]. This parameter has been studied extensively [41], but generally a lack of correlation between plasma DNA concentration and tumor size, stage or location has been reported.
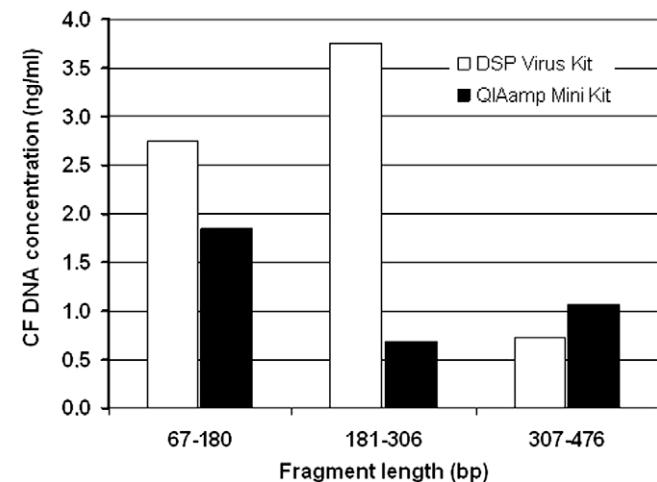
4.2. DNA integrity

DNA integrity is a parameter of interest for plasma CF DNA, since it could be used to extrapolate the origin of circulating DNA. As stated in the introduction, the dimension of DNA fragments is related to the mechanism of release from the cells. This

aspect can represent an useful parameter also in the view to clarify this problem. Few papers compared the DNA integrity index of cancer patients with that of healthy subjects, generally leading to the common conclusion that circulating DNA from cancer patients possesses a higher integrity index than the control population [57–63]. On the other hand, no difference was found between benign and malignant tumors [64,65]. The design of the assays differs significantly in regard to the target genes (ALU repeats, hTERT, ...) but shares the same principle for assay design, based on the use of a common forward primer and reverse primers delimitating amplicons of increasing dimension. The ratio between the quantity of the long versus the short amplicon represents the so called integrity index.

We built up an assay based on the amyloid precursor protein (APP) gene detection by qPCR, designing four different assays with comparable efficiency for the amplification of a 67, 180, 306 and 476 bp amplicon, respectively (unpublished data). This procedure allowed us to compare three integrity index (180/67, 306/67 and 476/67) to be used for the investigation of the circulating DNA integrity. Just to compare the extraction procedure we considered only healthy subjects whose plasma samples were submitted to the two different extraction procedures (DSP Virus Kit and QIAamp Mini Kit, Qiagen, Hilden, Germany). The results are reported in Fig. 1, where it can be evidenced that plasma total DNA concentration varies on the basis of the amplicon length. The two kits differ mainly when fragments between 67 and 306 bp are considered (Fig. 1). The apoptotic origin of the CF DNA fragments in healthy subjects seems to be confirmed by the evidenced higher concentration of fragments of dimensions comprised between 180 and 306 bp.

5. Cell-free DNA as a biomarker of cancer or disease

Most reports related to the diagnostic and prognostic applications of CF DNA are relative to neoplastic diseases. Nevertheless, the increase of circulating CF DNA is not a specific marker for malignant conditions. Plasma DNA levels are increased also in other physiological status and diseases, for example pregnancy and trauma. The detection in plasma/serum DNA of oncogene mutations, often encountered in a wide variety of cancer tissues, provides concrete evidence that CF nucleic acids are released into the circulation by tumors [47]. Thus, methods for the detection of tumor-specific DNA variants have been developed.

The main limitation to the molecular diagnosis of solid tumors is the frequent need for invasive procedures to obtain adequate testing materials [66]. So if, from a theoretical point of view, CNA are well accepted as non-invasive markers readily available in any stage of the disease or at any time in the follow up period, their low concentration in plasma limits their use and represents a way of selection for the methods to be used for detection and quantification. During the last 10 years there was a growing interest in circulating DNA, as demonstrated by the increasing number of publications. The analytical requirements of the methods used to study circulating DNA are so demanding for a strict specificity and a high sensitivity that the ideal method is still far to be found. Obviously many attempts are ongoing in designing new methods able to overcome the sensitivity limitation of conventional PCR and sequencing methods, but the number of publications already available in the field is so large that representative papers will be chosen for discussion. The mutation analysis on DNA extracted from plasma was related to the detection of variants in the K-ras oncogene. In fact it was chosen as a tumor specific marker, since it shows a very high mutation frequency. K-ras mutations are precocious events in neoplastic development starting from codons 12, 13 and 61 and detectable with high specificity and sensitivity in patients affected by colorectal cancer [67,68]. It was reported an 83% correspondence between the results found in plasma with those obtained from tissue [38] thus bringing to the definition of plasma as a surrogate sample for tumor tissue. Moreover, some patients showed mutated DNA in the CF compartment also in the absence of an evident neoplastic disease and this could represent a risk factor for tumor development [69]. K-ras DNA in plasma was studied also for the diagnosis of other cancers. The results did not show any significant relationship with the development of non small cell lung cancer (NSCLC), failing to confirm K-ras as a marker of tumor presence in this cancer type [70]. On the contrary, K-ras mutations were detected in patients with pancreatic cancer 5–14 months before tumor diagnosis and were absent in patients with benign pancreas pathologies and in healthy subjects [71].

As a signature of tumor-derived DNA, mutations in oncogenes and tumor suppressor genes can be used as tumor biomarkers. Besides K-ras mutation, the detection of common p53 mutations in plasma of smokers without cancer but not in the plasma of non-smokers indicated that the presence of p53 mutations in plasma DNA could reflect the exposure to carcinogens and, hence, the chance of developing lung cancer [72].

Other indicators of the presence of tumor-deriving DNA have been adopted such as microsatellite alterations [73–75], viral DNA [76], hypermethylation of tumor suppressor gene [77].

6. Cell-free RNA in cancer

When dealing with circulating RNA two different aspects must be considered: RNA can be cell-associated or cell-free.

The significance of mRNA deriving from cells has been widely investigated in the field of oncology with the aim to detect circulating tumor cells (CTCs). As there are no disease-specific markers uniquely associated with any solid cancer [78], the presence of CTCs in the blood stream has been assessed by indirect measurements of tissue-specific transcripts by RT-qPCR (reverse transcription-quantitative real-time PCR). However, the above aspect will not be considered in this paper which is focused on CF RNA.

The presence of CF RNA in plasma has been demonstrated by Lo et al. [79] as well as Kopresky et al. in 1999 [40]. Since then, the presence of tumor associated CF RNA has been detected in the plasma/serum of subjects affected by various cancers such as

Fig. 1. Cell-free DNA concentration in plasma from healthy donors ($n = 15$). On the basis of the amplicon size used by the real-time PCR methods, it was possible to calculate the amount of cell-free DNA (ng/ml of plasma) of DNA fragments of dimension comprised in the following ranges: (i) 67–180 bp; (ii) 181–306 bp and (iii) 307–476. Measurement of DNA concentrations were performed following two different type of extraction procedures: DSP Virus Kit (white columns) and QIAamp Mini Kit (black columns).

breast [80], colon [81] and lung [82], hepatocellular [83] and prostatic [84] carcinoma, gastric cancer [85] by qualitative PCR approaches or RT-qPCR [41].

The stability of RNA in plasma has been studied leading to the discovery that RNA is protected from RNase degradation being particle-associated [86]. These findings have been recently confirmed by Garcìa et al. [87] who also showed that this RNA fraction is enriched in mRNAs and small RNAs, suggesting a mechanism of active release from cells.

7. Cell-free RNA in pregnancy

The discovery of circulating fetal RNA in plasma of pregnant women [88] opened new perspectives in the research of new methods for non-invasive prenatal diagnosis. The source of fetal CF RNA in maternal circulation is represented by hematopoietic cells and the placenta [89].

The first detection of placental mRNAs in maternal plasma was reported by Ng et al. [90] who developed RT-qPCR methods specific for mRNA transcripts of the genes coding for human placental lactogen (hPL) and the beta subunit of human chorionic gonadotropin (βhCG).

Placental mRNA markers (hPL, βhCG, corticotropin-releasing hormone, tissue factor pathway inhibitor 2, KISS1, and placenta-specific protein 1) to be used in non-invasive prenatal gene expression profiling were identified by a micro-array based approach by Tsui et al. [91] and subsequently confirmed by RT-qPCR.

A preponderance of 5′mRNA fragments in circulating placental RNA was later observed, with potentially interesting implications for the development of new markers to be used in prenatal diagnosis [92].

The discovery of the presence of placental CF RNA since the 4th week of gestation [93] has confirmed the important role that can be played by this target in prenatal screening.

A great input in the field of placental CF RNA has been given by the study of markers related to disorders associated to pregnancy, in particular preeclampsia. Starting from the demonstration of increased levels of corticotropin-releasing hormone (CRH) mRNA in preeclampsia, compared with non preeclamptic pregnancies matched for gestational age [94], a series of markers were investigated, such as leptin [95] and urocortin [96].

In the first trimester of pregnancy circulating βhCG mRNA concentrations resulted significantly lower in the serum of mothers carrying fetuses with trisomy of chromosome 18 than in that of mothers with euploid fetuses [97]. Another approach to the same issue was carried out by Lo et al. [98] who investigated trisomy 21 fetuses by assessing the ratio between alleles of a single-nucleotide polymorphism (SNP) in the placenta-specific 4 (PLAC4) mRNA expressed by the placenta. The relative abundance of the two SNP alleles in heterozygous fetuses was determined by primer extension and mass spectrometry [98]. Recently [99] the principles of digital PCR have been applied to the detection of fetal chromosomal aneuploidy, measuring the allelic ratio of PLAC4 mRNA in maternal plasma. Digital PCR involves multiple PCR analyses on extremely diluted nucleic acids (one template molecule per reaction well): the proportion of positive amplifications among the total number of PCRs analyzed allows an estimation of the template concentration in the original non diluted sample.

8. An update on methodology

The most diffuse and suitable technique used to study CNA is PCR in its different applications.

To evidence tumor-associated mutations in the plasma/serum of cancer patients allele-specific polymerase chain reaction is the method of choice to detect mutated sequence present at a low concentration among a background of wild-type sequences [100,101].

This approach will require the design of allele-specific primers and hence the sequence information of the mutation and it would miss the tumor-associated variants occurring outside the mutation hot spots. Moreover, often the sensitivity characteristics of the method are not sufficient to detect the presence of the mutation in plasma. Alternative methods can be represented by emerging mass spectrometry-based method [102], but they do not solve all the above cited problems. For the identification of low level somatic mutations within wild-type DNA, a new PCR method has been reported named COLD-PCR [103]. It can be used in conjunction with high resolution melting analysis (HRMA) [104] improving the mutation scanning potency of this technique. It shows an increase in assay specificity and sensitivity over the conventional PCR, reaching detection limits of 2% of mutated allele in wild-type DNA [104].

Among the most useful technologies to be used in this field real-time PCR appears to be one of the best candidates to solve most of the problems related to post-PCR handling, assay sensitivity and specificity. In fact, it was reported as a method particularly suited for plasma mutation detection and quantification, since it reduces the chance of contamination and it can make use of molecules enhancing the discriminating power of the assay. For example, peptide nucleic acid (PNA) clamping and a locked nucleic acid (LNA) hybrid probe have been used to detect a single point mutation in plasma [105] and LNA oligonucleotides, that competitively inhibit primer binding to wild-type DNA, were adopted for selective amplification of rare mutations [106].

A COLD-real time approach has also been reported [107] and it can be reasonably considered up to now one of the techniques with the highest sensitivity and specificity to be used for rare mutated species. Anyway, COLD-PCR methods require a big effort for discovering the critical temperature which allows the preferential amplification of the mutated allele and moreover it can be applied to the detection of known variants.

9. Intercommunication via circulating nucleic acids

The intercellular exchange of genetic information between eukaryotic cells has been excluded since the publication of recent papers reporting on nanotubes, exosomes, apoptotic bodies, and nucleic acid – binding peptides that provide novel pathways for cell – cell communication, with implications in health and disease [108,109].

Functional delivery of mRNAs and micro-RNAs between mammalian cells has been demonstrated and this particular RNA was called "exosomal shuttle RNA" (esRNA) [110] to underline its specific function. Following this new approach CNAs are now under study for their possible function in intercellular signaling during development [111], in epigenetic remodeling [112], tissue regeneration and fine tuning of the adaptive immune system [113]. They may also be involved in cancer development and immune surveillance [113].

As far as cancer is concerned, nucleic acid transfer would enable malignant cells to influence the surrounding non-malignant cells and microenvironment in a highly specific and complex manner to assist the tumor in nutrient supply, invasion and metastasis [108]. Analogously, Al-Nedawi et al. [114] hypothesized that different clones of the same tumor can promote neoplastic proliferation by the exchanging of RNA-based signals.

Researchers in this field believe that this new approach to the understanding of cell-to-cell communication can strongly influence the way we treat various diseases [108].

10. Conclusions

We are optimistic that circulating nucleic acid analysis will become an important tool for the clinical management of cancer patients in the near future and that it could be used successfully for prenatal diagnosis even if, despite a great interest in this field and ample possibilities, results are contradictory and confusing [115].

Notwithstanding the lack of a precise knowledge on the origin and function of CNA, many researchers are actively working on the discovery of sensitive and specific markers in plasma/serum based on the detection of DNA variants or anomalous RNA expression able to evidence the presence of pathological conditions, especially in neoplastic diseases and pregnancy. The involvement in studies related to CNA derives from the conviction they represent a non-invasive tool for disease screening and for the early detection of pathological conditions. We are confident that new analytical principles, innovative methods with extraordinary sensitivity and specificity features, pre-analytical suitable procedures and standardized protocols for sample collection and analysis will be implemented in the next future. A deeper understanding of the biology of CNA can derive from an advance in the methodological and technological support to the study of these molecules.

References

[1] P. Mandel, P. Metais, C. R. Acad. Sci. Paris 142 (1948) 241–243.
[2] S.A. Leon, B. Shapiro, D.M. Sklaroff, M.J. Yaros, Cancer Res. 37 (1977) 646–650.
[3] E. Gormally, E. Caboux, P. Vineis, P. Hainaut, Mutat. Res. 635 (2007) 105–117.
[4] G. Goebel, M. Zitt, M. Zitt, H.M. Müller, Dis. Markers 21 (2005) 105–120.
[5] P. Anker, H. Mulcahy, X.Q. Chen, M. Stroun, Cancer Metastasis Rev. 18 (1999) 65–73.
[6] A. Rhodes, S.J. Wort, H. Thomas, P. Collinson, E.D. Bennett, Crit. Care 10 (2006) R60.
[7] M. Stroun, J. Lyautey, C. Lederrey, A. Olson-Sand, P. Anker, Clin. Chim. Acta 313 (2001) 139–142.
[8] Z. Chen, A. Fadiel, F. Naftolin, K.D. Eichenbaum, Y. Xia, Med. Hypotheses 65 (2005) 956–961.
[9] M. Frattini, D. Balestra, P. Verderio, G. Gallino, E. Leo, G. Sozzi, M.A. Pierotti, M.G. Daidone, J. Clin. Oncol. 23 (2005) 3163–3164.
[10] S. Gal, C. Fidler, Y.M. Lo, M. Taylor, C. Han, J. Moore, A.L. Harris, J.S. Wainscoat, Br. J. Cancer 90 (2004) 1211–1215.
[11] H. Schwarzenbach, K. Pantel, B. Kemper, C. Beeger, F. Otterbach, R. Kimmig, S. Kasimir-Bauer, Breast Cancer Res. 11 (5) (2009) R71.
[12] H. Schwarzenbach, C. Alix-Panabières, I. Müller, N. Letang, J.P. Vendrell, X. Rebillard, K. Pantel, Clin. Cancer Res. 15 (3) (2009) 1032–1038.
[13] S. Hahn, L.G. Jackson, V. Kolla, A.P. Mahyuddin, M. Choolani, Expert Rev. Mol. Diagn. 9 (6) (2009) 613–621.
[14] E.C. Hung, R.W. Chiu, Y.M. Lo, J. Clin. Pathol. 62 (2009) 308–313.
[15] V. Swarup, M.R. Rajeswar, FEBS Lett. 581 (2007) 795–799.
[16] Y.M. Lo, J. Zhang, T.N. Leung, T.K. Lau, A.M. Chang, N.M. Hjelm, Am. J. Hum. Genet. 64 (1999) 218–224.
[17] T. Tsumita, M. Iwanaga, Nature 198 (1963) 1088–1089.
[18] P. Rumore, B. Muralidhar, M. Lin, C. Lai, C.R. Steinman, Clin. Exp. Immunol. 90 (1992) 56–62.
[19] S. Jahr, H. Hentze, S. Englisch, D. Hardt, F.O. Fackelmayer, R.D. Hesch, R. Knippers, Cancer Res. 61 (2001) 1659–1665.
[20] Y.M.D. Lo, R.W.K. Chiu, Clin. Chem. 55 (2009) 607–608.
[21] M. van der Vaart, P.J. Pretorius, Ann. NY Acad. Sci. 1137 (2008) 92–97.
[22] J. Beck, H.B. Urnovitz, J. Riggert, M. Clerici, E. Schütz, Clin. Chem. 55 (2009) 730–738.
[23] M. van der Vaart, D.V. Semenov, E.V. Kuligina, V.A. Richter, P.J. Pretorius, Clin. Chim. Acta 409 (2009) 21–27.
[24] F. Diehl, M. Li, D. Dressman, Y. He, D. Shen, S. Szabo, L.A. Diaz Jr., S.N. Goodman, K.A. David, H. Juhl, K.W. Kinzler, B. Vogelstein, Proc. Natl. Acad. Sci. USA 102 (2005) 16368–16373.
[25] D. García-Olmo, D.C. García-Olmo, Ann. NY Acad. Sci. 945 (2001) 265–275.
[26] D.C. García-Olmo, R. Ruiz-Piqueras, D. García-Olmo, Histol. Histopathol. 19 (2004) 575–583.
[27] M.D. Teare, P.J. Woll, Expert Rev. Mol. Diagn. 7 (2007) 699–702.
[28] M.A. Thijssen, D.W. Swinkels, T.J. Ruers, J.B. de Kok, Anticancer Res. 22 (2002) 421–425.
[29] G. Sozzi, L. Roz, D. Conte, L. Mariani, F. Andriani, P. Verderio, U. Pastorino, J. Natl. Cancer Inst. 97 (2005) 1848–1850.
[30] T.H. Lee, L. Montalvo, V. Chrebtow, M.P. Busch, Transfusion 41 (2001) 276–282.
[31] Y.M. Lo, M.S. Tein, T.K. Lau, C.J. Haines, T.N. Leung, P.M. Poon, J.S. Wainscoat, P.J. Johnson, A.M. Chang, N.M. Hjelm, Am. J. Hum. Genet. 62 (1998) 768–775.
[32] M. Jung, S. Klotzek, M. Lewandowski, M. Fleischhacker, K. Jung, Clin. Chem. 49 (2003) 1028–1029.
[33] N.Y. Lam, T.H. Rainer, R.W. Chiu, Y.M. Lo, Clin. Chem. 50 (2004) 256–257.
[34] P.W. Chiang, D.G. Beer, W.L. Wei, M.B. Orringer, D.M. Kurnit, Clin. Cancer Res. 5 (1999) 1381–1386.
[35] R.W. Chiu, L.L. Poon, T.K. Lau, T.N. Leung, E.M. Wong, Y.M. Lo, Clin. Chem. 47 (2001) 1607–1613.
[36] D.W. Swinkels, E. Wiegerinck, E.A. Steegers, J.B. de Kok, Clin. Chem. 49 (2003) 525–526.
[37] Y.Y. Lui, K.W. Chik, Y.M. Lo, Clin. Chem. 48 (2002) 2074–2076.
[38] M.S. Kopreski, F.A. Benko, C. Kwee, K.E. Leitzel, E. Eskander, A. Lipton, C.D. Gocke, Br. J. Cancer 76 (1997) 1293–1299.
[39] T. Lee, E.S. LeShane, G.M. Messerlian, J.A. Canick, A. Farina, W.W. Heber, D.W. Bianchi, Am. J. Obstet. Gynecol. 187 (2002) 1217–1221.
[40] M.S. Kopreski, F.A. Benko, L.W. Kwak, C.D. Gocke, Clin. Cancer Res. 5 (1999) 1961–1965.
[41] M. Fleischhacker, B. Schmidt, Biochim. Biophys. Acta 1775 (2007) 181–232.
[42] C. Kirsch, S. Weickmann, B. Schmidt, M. Fleischhacker, Ann. NY Acad. Sci. 1137 (2008) 135–139.
[43] R.E. Board, V.S. Williams, L. Knight, J. Shaw, A. Greystoke, M. Ranson, C. Dive, F.H. Blackhall, A. Hughes, Ann. NY Acad. Sci. 1137 (2008) 98–107.
[44] F.B. Clausen, G.R. Krog, K. Rieneck, M.H. Dziegiel, Prenat. Diagn. 27 (2007) 6–10.
[45] N.B. Tsui, E.K. Ng, Y.M. Lo, Clin. Chem. 48 (2002) 1647–1653.
[46] T. Dovc-Drnovsek, B. Emersic, P. Rozman, D. Cerne, J. Lukac-Bajalo, Ann. NY Acad. Sci. 1137 (2008) 125–129.
[47] K.C. Chan, Y.M. Lo, Histol. Histopathol. 17 (2002) 937–943.
[48] M. van der Vaart, P.J. Pretorius, Ann. NY Sci. 1137 (2008) 18–26.
[49] M. Stroun, P. Anker, J. Lyautey, C. Lederrey, P.A. Maurice, Eur. J. Cancer Clin. Oncol. 23 (1987) 707–712.
[50] G. Sozzi, D. Conte, L. Mariani, V.S. Lo, L. Roz, C. Lombardo, M.A. Pierotti, L. Tavecchio, Cancer Res. 61 (2001) 4675–4678.
[51] C.R. Steinman, J. Clin. Invest. 56 (1975) 512–515.
[52] J.M. Silva, J. Silva, A. Sanchez, J.M. Garcia, G. Dominguez, M. Provencio, L. Sanfrutos, E. Jareno, A. Colas, P. Espana, F. Bonilla, Clin. Cancer Res. 8 (2002) 3761–3766.
[53] B. Taback, S.J. O'Day, D.S. Hoon, Ann. NY Acad. Sci. 1022 (2004) 17–24.
[54] G. Sozzi, D. Conte, M. Leon, R. Ciricione, L. Roz, C. Ratcliffe, E. Roz, N. Cirenei, M. Bellomi, G. Pelosi, M.A. Pierotti, U. Pastorino, J. Clin. Oncol. 21 (2003) 3902–3908.
[55] H.W. Chang, S.M. Lee, S.N. Goodman, G. Singer, S.K. Cho, L.J. Sokoll, F.J. Montz, R. Roden, Z. Zhang, D.W. Chan, R.J. Kurman, I. Shih, J. Natl. Cancer Inst. 94 (2002) 1697–1703.
[56] S. Sifakis, A. Zaravinos, N. Maiz, D.A. Spandidos, K.H. Nicolaides, Am. J. Obstet. Gynecol. 201 (2009) 472.e1–472.e7.
[57] B.G. Wang, H.Y. Huang, Y.C. Chen, R.E. Bristow, K. Kassauei, C.C. Cheng, R. Roden, L.J. Sokoll, D.W. Chan, IeM. Shih, Cancer Res. 63 (2003) 3966–3968.
[58] N. Suzuki, A. Kamataki, J. Yamaki, Y. Homma, Clin. Chim. Acta 387 (2008) 55–58.
[59] K.C. Chan, J. Zhang, A.B. Hui, N. Wong, T.K. Lau, T.N. Leung, K.W. Lo, D.W. Huang, Y.M. Lo, Clin. Chem. 50 (2004) 88–92.
[60] R.E. Board, R.E. Board, V.S. Williams, L. Knight, J. Shaw, A. Greystoke, M. Ranson, C. Dive, F.H. Blackhall, A. Hughes, Ann. NY Acad. Sci. 1137 (2008) 98–107.
[61] N. Umetani, A.E. Giuliano, S.H. Hiramatsu, F. Amersi, T. Nakagawa, S. Martino, D.S. Hoon, J. Clin. Oncol. 24 (2006) 4270–4276.
[62] W.W. Jiang, M. Zahurak, D. Goldenberg, Y. Milman, H.L. Park, W.H. Westra, W. Koch, D. Sidransky, J. Califano, Int. J. Cancer 119 (2006) 2673–2676.
[63] N. Umetani, J. Kim, S. Hiramatsu, H.A. Reber, O.J. Hines, A.J. Bilchik, D.S. Hoon, Clin. Chem. 52 (2006) 1062–1069.
[64] S. Holdenrieder, A. Burges, O. Reich, F.W. Spelsberg, P. Stieber, Ann. NY Acad. Sci. 1137 (2008) 162–170.
[65] B. Schmidt, S. Weickmann, C. Witt, M. Fleischhacker, Ann. NY Sci. 1137 (2008) 207–213.
[66] A. Ziegler, U. Zangemeister-Wittke, R.A. Stahel, Cancer Treat. Rev. 28 (2002) 255–271.
[67] Z. Ronai, T. Minamoto, R. Butler, M. Tobi, F.Q. Luo, E. Zang, H. Esumi, T. Sugimura, Cancer Detect. Prev. 19 (1995) 512–517.
[68] G.D. Sorenson, D.M. Pribish, F.H. Valone, V.A. Memoli, D.J. Bzik, S.L. Yao, Cancer Epidemiol. Biomarkers Prev. 3 (1994) 67–71.
[69] M.S. Kopreski, F.A. Benko, D.J. Borys, A. Khan, T.J. McGarrity, C.D. Gocke, J. Natl. Cancer Inst. 92 (2000) 918–923.
[70] S. Trombino, M. Neri, R. Puntoni, C. Angelini, M. Loprevite, A. Cesario, P. Granone, A. Imperatori, L. Dominioni, A. Ardizzoni, R. Filiberti, P. Russo, Clin. Chem. 51 (2005) 1313–1314.
[71] H.E. Mulcahy, J. Lyautey, C. Lederrey, X. qi Chen, P. Anker, E.M. Alstead, A. Ballinger, M.J. Farthing, M. Stroun, Clin. Cancer Res. 4 (1998) 271–275.
[72] N. Hagiwara, L.E. Mechanic, G.E. Trivers, H.L. Cawley, M. Taga, E.D. Bowman, K. Kumamoto, P. He, M. Bernard, S. Doja, M. Miyashita, T. Tajiri, K. Sasajima, T. Nomura, H. Makino, K. Takahashi, S.P. Hussain, C.C. Harris, Cancer Res. 66 (2006) 8309–8317.
[73] C.F. Eisenberger, N.H. Stoecklein, S. Jazra, S.B. Hosch, M. Peiper, P. Scheunemann, J.S. Am Esch, W.T. Knoefel, Eur. J. Surg. Oncol. 32 (2006) 954–960.
[74] X.Q. Chen, M. Stroun, J.L. Magnenat, L.P. Nicod, A.M. Kurt, J. Lyautey, C. Lederrey, P. Anker, Nat. Med. 2 (1996) 1033–1035.

[75] J. Li, L. Harris, H. Mamon, M.H. Kulke, W.H. Liu, P. Zhu, G.M. Makrigiorgos, J. Mol. Diagn. 8 (2006) 22–30.
[76] J.C. Tsang, Y.M. Lo, Pathology 39 (2007) 197–207.
[77] R.E. Board, L. Knight, A. Greystoke, F.H. Blackhall, A. Hughes, C. Dive, M. Ranson, Biomark. Insights 2 (2007) 307–319.
[78] S.A. Bustin, R. Mueller, Clin. Sci. (Lond.) 109 (2005) 365–379.
[79] K.W. Lo, Y.M. Lo, S.F. Leung, Y.S. Tsang, L.Y. Chan, P.J. Johnson, N.M. Hjelm, J.C. Lee, D.P. Huang, Clin. Chem. 45 (1999) 1292–1294.
[80] X.Q. Chen, H. Bonnefoi, M.F. Pelte, J. Lyautey, C. Lederrey, S. Movarekhi, P. Schaeffer, H.E. Mulcahy, P. Meyer, M. Stroun, P. Anker, Clin. Cancer Res. 6 (2000) 3823–3826.
[81] J.M. Silva, R. Rodríguez, J.M. García, C. Muñoz, J. Silva, G. Domínguez, M. Provencio, P. España, F. Bonilla, Gut 50 (2002) 530–534.
[82] M. Fleischhacker, T. Beinert, M. Ermitsch, D. Seferi, K. Possinger, C. Engelmann, B. Jandrig, Ann. NY Acad. Sci. 945 (2001) 179–188.
[83] N. Miura, G. Shiota, T. Nakagawa, Y. Maeda, A. Sano, A. Marumoto, Y. Kishimoto, Y. Murawaki, J. Hasegawa, Oncology 64 (2003) 430–434.
[84] E. Papadopoulou, E. Davilas, V. Sotiriou, A. Koliopanos, F. Aggelakis, K. Dardoufas, N.J. Agnanti, I. Karydas, G. Nasioulas, Oncol. Res. 14 (2004) 439–445.
[85] N. Tani, D. Ichikawa, D. Ikoma, H. Tomita, S. Sai, H. Ikoma, K. Okamoto, T. Ochiai, Y. Ueda, E. Otsuji, H. Yamagishi, N. Miura, G. Shiota, Anticancer Res. 27 (2007) 1207–1212.
[86] E.K. Ng, N.B. Tsui, N.Y. Lam, R.W. Chiu, S.C. Yu, S.C. Wong, E.S. Lo, T.H. Rainer, P.J. Johnson, Y.M. Lo, Clin. Chem. 48 (2002) 1212–1217.
[87] J.M. García, V. García, C. Peña, G. Domínguez, J. Silva, R. Diaz, P. Espinosa, M.J. Citores, M. Collado, F. Bonilla, RNA 14 (2008) 1424–1432.
[88] L.L. Poon, T.N. Leung, T.K. Lau, Y.M. Lo, Clin. Chem. 46 (2000) 1832–1834.
[89] J.L. Maron, D.W. Bianchi, Am. J. Med. Genet. C Semin. Med. Genet. 145C (2007) 5–17.
[90] E.K.O. Ng, N.B.Y. Tsui, T.K. Lau, T.N. Leung, R.W.K. Chiu, N.S. Panesar, L.C.W. Lit, K.W. Chan, Y.M.D. Lo, Proc. Natl. Acad. Sci. USA 100 (2003) 4748–4753.
[91] N.B.Y. Tsui, S.S.C. Chim, R.W.K. Chiu, T.K. Lau, E.K.O. Ng, T.N. Leung, Y.K. Tong, K.C.A. Chan, Y.M.D. Lo, J. Med. Genet. 41 (2004) 461–497.
[92] B.C.K. Wong, R.W.K. Chiu, N.B.Y. Tsui, K.C.A. Chan, L.W. Chan, T.K. Lau, T.N. Leung, Y.M.D. Lo, Clin. Chem. 51 (2005) 1786–1795.
[93] R.W. Chiu, W.B. Lui, M.C. Cheung, N. Kumta, A. Farina, I. Banzola, S. Grotti, N. Rizzo, C.J. Haines, Y.M. Lo, Clin. Chem. 52 (2) (2006) 313–316.
[94] E.K.O. Ng, T.N. Leung, N.B.Y. Tsui, T.K. Lau, N.S. Panesar, R.W.K. Chiu, Y.M.D. Lo, Clin. Chem. 49 (2003) 727–731.
[95] H. Laivuori, M.J. Gallaher, L. Collura, W.R. Crombleholme, N. Markovic, A. Rajakumar, C.A. Hubel, J.M. Roberts, R.W. Powers, Mol. Hum. Reprod. 12 (2006) 551–556.
[96] P. Florio, M. Torricelli, G. De Falco, E. Leucci, A. Giovannelli, D. Gazzolo, F.M. Severi, F. Bagnoli, L. Leoncini, E.A. Linton, F. Petraglia, J. Hypertens. 24 (2006) 1831–1840.
[97] E.K. Ng, A. El-Sheikhah, R.W. Chiu, K.C. Chan, M. Hogg, R. Bindra, T.N. Leung, T.K. Lau, K.H. Nicolaides, Y.M. Lo, Clin. Chem. 50 (2004) 1055–1057.
[98] Y.M. Lo, N.B. Tsui, R.W. Chiu, T.K. Lau, T.N. Leung, M.M. Heung, A. Gerovassili, Y. Jin, K.H. Nicolaides, C.R. Cantor, C. Ding, Nat. Med. 13 (2007) 218–223.
[99] Y.M. Lo, F.M. Lun, K.C. Chan, N.B. Tsui, K.C. Chong, T.K. Lau, T.Y. Leung, B.C. Zee, C.R. Cantor, R.W. Chiu, Proc. Natl. Acad. Sci. USA 104 (2007) 13116–13121.
[100] K.C. Chan, Y.M. Lo, Br. J. Cancer 96 (2007) 681–685.
[101] M. Yancovitz, J. Yoon, M. Mikhail, W. Gai, R.L. Shapiro, R.S. Berman, A.C. Pavlick, P.B. Chapman, I. Osman, D. Polsky, J. Mol. Diagn. 9 (2007) 178–183.
[102] C. Ding, R.W.K. Chiu, T.K. Lau, T.N. Leung, L.C. Chan, A.Y. Chan, P. Charoenkwan, I.S. Ng, H.Y. Law, E.S. Ma, X. Xu, C. Wanapirak, T. Sanguansermsri, C. Liao, M.A. Ai, D.H. Chui, C.R. Cantor, Y.M.D. Lo, Proc. Natl. Acad. Sci. USA 101 (2004) 10762–10767.
[103] J. Li, G.M. Makrigiorgos, Biochem. Soc. Trans. 37 (2009) 427–432.
[104] C.A. Milbury, G.M. Makrigiorgos, Clin. Chem. 55 (2009) 2130–2143.
[105] M. Shinozaki, S.J. O'Day, M. Kitago, F. Amersi, C. Kuo, J. Kim, H.J. Wang, D.S. Hoon, Clin. Cancer Res. 13 (2007) 2068–2074.
[106] R.P. Oldenburg, M.S. Liu, M.S. Kolodney, J. Invest. Dermatol. 128 (2008) 398–402.
[107] J. Li, L. Wang, P.A. Jänne, G.M. Makrigiorgos, Clin. Chem. 55 (2009) 748–756.
[108] M. Belting, A. Wittrup, J. Cell Biol. 183 (2008) 1187–1191.
[109] A. Wittrup, M. Belting, Methods Mol. Biol. 480 (2009) 101–112.
[110] H. Valadi, K. Ekström, A. Bossios, M. Sjöstrand, J.J. Lee, J.O. Lötvall, Nat. Cell Biol. 9 (2007) 654–659.
[111] J. Ratajczak, K. Miekus, M. Kucia, J. Zhang, R. Reca, P. Dvorak, M.Z. Ratajczak, Leukemia 20 (2006) 847–856.
[112] V.L. Chandler, Cell 128 (2007) 641–645.
[113] J.S. Schorey, S. Bhatnagar, Traffic 9 (2008) 871–881.
[114] K. Al-Nedawi, B. Meehan, J. Micallef, V. Lhotak, L. May, A. Guha, J. Rak, Nat. Cell Biol. 10 (2008) 619–624.
[115] M. van der Vaart, P.J. Pretorius, Clin. Biochem. (2009) (Epub ahead of print).