



Review

Circulating nucleic acids (CNAs) and cancer—A survey

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Received 28 March 2006; received in revised form 4 October 2006; accepted 4 October 2006

Available online 7 October 2006

Abstract

It has been known for decades that it is possible to detect small amounts of extracellular nucleic acids in plasma and serum of healthy and diseased human beings. The unequivocal proof that part of these circulating nucleic acids (CNAs) is of tumor origin, initiated a surge of studies which confirmed and extended the original observations. In the past few years many experiments showed that tumor-associated alterations can be detected at the DNA and RNA level. At the DNA level the detection of point mutations, microsatellite alterations, chromosomal alterations, i.e. inversion and deletion, and hypermethylation of promoter sequences were demonstrated. At the RNA level the overexpression of tumor-associated genes was shown. These observations laid the foundation for the development of assays for an early detection of cancer as well as for other clinical means.

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Keywords: Circulating nucleic acids; DNA; RNA; Plasma; Cancer; Review**Contents**

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1. What are CNAs—An introduction and short history

CNAs are extracellular nucleic acids found in cell-free plasma/serum of humans [1], plants [2] and in the animal

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kingdom [3]. There are several terms in use like circulating nucleic acids, extracellular nucleic acids or cell-free nucleic acids. We would like to propose to reserve the term circulating nucleic acids (CNA) for DNA and RNA obtained from plasma/serum and other “circulating” body fluids like lymphatic fluid. For nucleic acids obtained from body fluids like liquor, ascites, milk, bronchial lavage fluids, urine, stool, nucleic acids isolated from cell-free supernatants of in vitro cultivated cells or attached to the outside of the cell surface we will use the terms extracellular or cell-free nucleic acids (Fig. 1).

The first evidence for the existence of extracellular nucleic acids goes back to the observation made by Griffith who demonstrated the transmission of a pathogenic property from infectious bacteria which were heat killed to a live non-pathogenic strain [4]. A few years later Avery and coworkers demonstrated not only a transfer of genetic material between different *Pneumococcus* strains, but unequivocally proved, that DNA is the substance of inheritance, i.e. the “transforming principle” originally described by Griffith [5]. Later studies with *Bacillus subtilis* showed that the release of DNA is not the result of cell lysis but an active process linked to DNA synthesis [6]. Additional studies in plants and animals demonstrated the existence of circulating DNA in these kingdoms as well. In plants these nucleic acids are able to enter nuclei and can be integrated and expressed in these cells, leading to the concept of messenger DNA [7].

The detection of free-circulating nucleic acids in plasma samples of humans was reported for the first time by Mandel and Metais [1]. Apart from plasma and serum, extracellular nucleic acids can be isolated from liquor, ascites, milk, lymphatic and peritoneal fluids, bone-marrow aspirates, urine, prostatic fluid, bronchial lavage, sputum, cerebrospinal fluid, gastric and biliary juices, and even stool samples. The presence of tumor-associated alterations has been described in plasma/serum DNA from patients with cancers of the breast, cervix, colon, esophagus, stomach, head and neck, blood, liver, kidney, pancreas, skin, nasopharynx, ovary, prostate lung and thyroid.

2. Physico-chemical and biological characteristics of CNAs

Stroun and coworkers were the first to characterize plasma DNA, using a phenol extraction method, followed by a purification with a concanavalin-A column and ultracentrifuga-

tion [8]. The isolated material was shown to be double-stranded DNA, resistant to RNase and pronase, but digestible with DNase I and mainly of low molecular weight. The application of a test for the detection of DNA with a decreased strand stability (characteristic for tumor cell derived DNA) demonstrated that at least part of the DNA isolated from the plasma of cancer patients originates from the tumor cells [9]. Several electron microscopic and sucrose-gradient sedimentation analyses of free-circulating DNA showed fragments ranging in size from 500 bp up to >30 kb [10–12].

Nucleic acids released from cells are not “naked”, but can be part of complexes, or are bound to the surface of the cells via proteins with specific nucleic acid binding properties [13–16]. The cell membrane of erythrocytes and leukocytes seem to be loaded with nucleic acids (DNA and RNA), which can be eluted from these cells with a PBS/EDTA or trypsin treatment [17]. Additionally, CNAs are complexed with proteolipids [18,19], embedded into apoptotic vesicles [20], or complexed to the p53 protein [21]. Stroun et al. showed that nonstimulated human lymphocytes and frog auricles release a complex into the cultivation medium, in which the RNA and DNA are linked by hydrogen bonds [22]. The secretion of an RNA–proteolipid complex, isolated from sera of patients with malignant disorders and from tumor cell culture supernatants was also described [19,23–30]. Experiments in which cells were killed by mild heat treatment showed that the RNA release is dependent on intact and viable cells [22] and that the release of RNA and DNA into the cultivation medium is regulated by a homeostatic mechanism [6]. In contrast to these results, there is also a report in which no association between the DNA which is extruded by rat spleen cells in culture and any RNA was found [31].

Viruses which are released into the cellular environment are usually encapsidated but it was not clear whether Epstein–Barr Virus (EBV) DNA present in the plasma is “naked” or not. Zingg and coworkers obtained cell-free supernatants from an EBV-producing cell line and found that a DNase I digestion completely destroyed the β -actin encoding (i.e. cellular) DNA, but left the EBV DNA intact [32]. A comparable observation was described by others, who evaluated serum samples from EBV DNA positive nasopharyngeal carcinoma (NPC) patients after DNase I treatment and found that a PCR did not yield any product of the control β -globin DNA, whereas a PCR for EBV DNA remained positive in more than half of the samples [33]. In contrast, others did not find amplifiable β -globin and EBV DNA in any of the NPC and Hodgkin disease (HD) samples after DNase I digestion [34,35]. Additionally, the concentration of detectable EBV DNA dropped substantially when the amplicon size was increased (82 to 181 bp) with almost no PCR product when the size was larger than 385 bp, leading the authors to conclude that the viral DNA is naked in both patient populations.

EBV DNA was almost always found naked in the plasma of AIDS-related lymphoma patients and immunosuppressed/posttransplantation patients, while infectious mononucleosis patients often had a mixture of encapsidated and naked EBV DNA [36]. A real-time PCR targeting genomic β -globin sequences as a control gene demonstrated an efficient

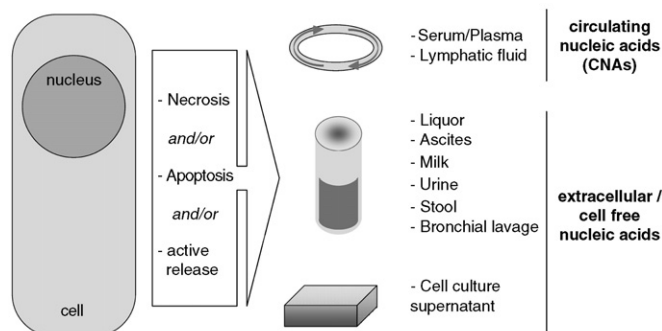


Fig. 1. Possible mechanism of release for extracellular nucleic acids.

degradation of the β -globin and EBV DNA in all of the AIDS-related lymphoma and post-transplantation lymphoproliferative disorder patients, whereas in the infectious mononucleosis plasma samples the β -globin DNA was not completely degraded.

These conflicting results could be interpreted in a way, that the release mechanism/s of virus particles and “naked” viral DNA is variable in different patient populations, but more work is needed to clarify this point.

3. Quantification of free-circulating nucleic acids

The first observation that the median DNA concentration in the serum of tumor patients is higher than in normal subjects was published by Leon et al. [37]. The data summarized in Table 1 (see www.cnaps.net) demonstrate that most of the normal subjects have a low plasma DNA concentration in comparison to the increased DNA values found in many cancer patients, but it is also obvious, that there is an overlap. An increased plasma DNA level is not only seen in tumor patients, but has been observed in patients with premalignant states, inflammation, trauma, after exhaustive exercise and in elderly patients suffering from acute or chronic illnesses [38–46]. In a large prospective study, sera from 1184 cancer-free persons living across Europe were analyzed and the only association found was between DNA quantity and death from chronic obstructive pulmonary disease (COPD) [47]. An elevated plasma DNA level was observed in 71% of the patients with lung cancer, however in 37% of the patients with benign pulmonary diseases as well [48]. When the plasma DNA concentration in 100 lung cancer patients was compared with 100 matched control subjects, a significant difference in plasma DNA level was described [49]. As interesting as these observations are, other authors showed that there is no correlation between plasma DNA concentration, and tumor size, stage or location and that an increased plasma DNA concentration can be observed at an early stage of the tumor development [42,46,48,50,51].

Therefore it is necessary to analyze the kinetics of the concentration of free circulating DNA. The level of plasma DNA is not constant but fluctuates, as was demonstrated when free-circulating DNA from two patients obtained at different time points in the course of the disease were quantified and an increased amount of plasma DNA was observed as the tumor growth progressed [9]. Such a fluctuation of maternal and fetal DNA was also observed in pregnant women, in which the variation in the amount of maternal cell-free DNA over 3 days was on average 21.5-fold, whereas the mean variation in cell-free fetal DNA levels was only 2.2-fold [52]. This led to the question, whether tumor derived and normal extracellular DNA in cancer patients behaves the same way. In contrast to these observations no fluctuation of the total amount of CNAs was found when the quantity of plasma DNA isolated from patients (obtained on 3 consecutive days) with intestinal polyps or colorectal cancer (CRC) was analyzed [53]. Since a single quantitative assessment of CNAs will very likely be of limited value, other markers have to be included to make an analysis

clinically relevant [54]. This will hold true even if assays for DNA quantification are developed which are simple, rapid and sensitive [55].

It was shown that a therapy response is associated with a decrease in the plasma DNA level, whereas no change or even an increase in the amount of CNAs is seen in patients who do not respond to a therapy [37,48,49,51]. Patients with non small-cell lung cancer (NSCLC) showed a significant correlation between an increased plasma DNA concentration and tumor progression after chemotherapy treatment [50]. But there are also reports, in which no correlation between the level of circulating CNAs and any clinicopathological data were found [56,57]. In one study the serum and plasma DNA levels of CRC patients did not correlate and each value had a different correlation with the diagnosis and prognosis. While the serum DNA concentration was significantly associated with the presence of liver metastases, the plasma DNA was predictive for disease recurrence only [58]. In another study the cell-free DNA from pleural fluid was quantified, and it was concluded that it is possible to differentiate between exudative and transudative effusions [59].

The concept of “long DNA” is based on the observation that the fecal DNA isolated from patients with a colorectal tumor had a higher molecular weight than from cancer-free patients [60]. Assuming that this observation might hold true for CNAs as well, the length of plasma DNA isolated from tumor and non-tumor patients was compared [61]. A qualitative PCR using the β -actin gene as target was established with amplicon sizes of 100 bp and 400 bp length. The authors found longer DNA amplicons in tumor patients and concluded, that it is possible to differentiate between patients with benign and malignant disease. We established a quantitative real-time assay, using the human endogenous retrovirus gene as a target and did not find any differences between patients with a benign lung disease and patients with a lung tumor (unpublished observations). When the length of free-circulating DNA in a small group of CRC patients was analyzed, 2/3 patients with stage D showed no increase in the quantity of DNA if the amplicon size was reduced from approximately 1300 bp to 100 bp [62], but when the same experiment was repeated for the detection of mutated APC fragments, the increase varied from 10 to approximately 35-fold, leading to the question whether DNA derived from normal and tumor cells behaves differently.

An elevated concentration of nucleosomes might be a good apoptosis marker in tumor patients and an increased quantity was found in more than 53% of the lung cancer patients. Also an association between plasma DNA concentration, lactate dehydrogenase (LDH) and neuron-specific enolase (NSE) concentration, disease stage and survival was observed [40]. Holdenrieder et al. used a DNA-ELISA assay to compare the quantity of circulating nucleosomes in healthy subjects, tumor patients and patients with an acute inflammation [63]. It had also been shown that the decrease in the nucleosome level after the therapy was often correlated with a shrinkage of the tumor, suggesting that nucleosome measurement might be useful as a marker for prognostic purposes [64,65], and a similar

Table 1
Nucleic acid quantification in plasma and serum of healthy subjects, patients with benign and malignant diseases

Patient population	DNA in plasma	DNA in serum	RNA in plasma	Quantification method	References/remarks
10 patients with benign prostatic hypertrophy	936 genome equivalents/mL			quantitative real-time PCR	Allen et al. 2004 [315]
15 prostate adenocarcinoma patients	1734 genome equivalents/mL				
12 patients with high-grade prostatic intraepithelial neoplasia	1780 genome equivalents/mL				
25 healthy half-marathon runners	resting state: 18 pg/ μ L immediately after race: 334 pg/ μ L 2 h after race: 30 pg/ μ L			staining with Vistra Green and fluorescence measurement on a Light Cycler	Atermaniuk et al. 2004 [38]
33 lung cancer patients	5 to >125 ng/mL			Dipstick (Invitrogen)	Bearzatto et al. 2002 [316]
34 lung cancer patients	157 ng/mL			PicoGreen dsDNA Quantitation kit (Molecular Probes)	Beau-Faller et al. 2003 [56]
20 patients with benign lung disease	78 ng/mL				
56 stage I NSCLC survivors	30 ng/mL			spectrometry or DNA dip stick kit	Belinsky et al. 2005 [267]
121 smokers (40 pack years)	45 ng/mL				
74 never smokers	37 ng/mL				
20 leukaemia patients	5.4 μ g/mL			binding to labeled actinomycin D	Carpentier et al. 1981 [317]
44 healthy subjects	7 ng/mL			PicoGreen; dsDNA quantification kit	Chang et al. 2002 [128]
164 patients with non-malignant diseases	16 ng/mL				
122 unselected cancer patients	59 ng/mL				
15 ovarian cancer patients	821 ng/mL ascites fluid			PicoGreen dsDNA quantification kit	Chang et al. 2002 [133]
2 patients with colorectal carcinoma	101 ng/mL ascites fluid				
3 patients with pancreatic adenocarcinoma					
20 patients without any signs of malignancy					
117 HNSCC patients	100–688 ng/mL in 41 patients			fluorometric method based on Hoechst dye	Coulet et al. 2000 [57]
66 healthy subjects		318 ng/mL		solid-phase RIA	Cox et al. 1976 [318]
9 SLE patients		724 ng/mL			
13 rheumatoid arthritis patients		416 ng/mL			
10 malignant melanoma patients		2160 ng/mL			
12 breast cancer patients		549 ng/mL			
21 colorectal cancer patients		609 ng/mL			
Commercially obtained human serum samples		546 ng/mL		solid-phase RIA	Cox et al. 1976 [319] values for dog, horse goat, fetal calf, rabbit, guinea pig, rat, hamster and mouse ranged from 48 to 390 ng/mL, no DNA was found in cat serum,
66 controls	35 ng/mL (7 controls)	318 ng/mL		RIA	Cox et al. 1977 [320]

Table 1 (continued)

Patient population	DNA in plasma	DNA in serum	RNA in plasma	Quantification method	References/remarks
37 lymphoma patients	256 ng/mL			Spectrophotometry	Deligezer et al. 2003 [149]
20 healthy subjects	44 ng/mL				
5 healthy subjects	6 ng/mL			electron microscopy	Dennin et al. 1979 [10]
126 breast cancer patients	211 ng/mL			unknown	Di et al. 2003 [321]
92 healthy women	21 ng/mL				
11 adenoma patients	14 ng/mL			real-time quantitative PCR	Diehl et al. 2005 [62]
16 colorectal carcinoma patients stage A and B	12 ng/mL				
6 colorectal carcinoma patients stage D	158 ng/mL				
Unknown number of healthy subjects			1–10 ng/ μ L	quantitative real-time RT-PCR	El-Hefnawy et al. 2004 [322]
68 lung cancer patients (46 NSCLC, 22 SCLC)	30 ng/mL			slot dot hybridization after radiolabeling	Fournie et al. 1995 [40]
26 healthy subjects	10 ng/mL				
96 women with primary breast cancer		221 ng/mL		real-time PCR	Gal et al. 2004 [54]
24 healthy subjects		63 ng/mL			
46 healthy subjects	1.8 ng/mL	12.6 ng/mL		real-time PCR	Gautschi et al. 2004 [50]
173 NSCLC patients	3.7 ng/mL	36.9 ng/mL			
51 healthy subjects			144 μ g/mL	fluorometric method	Guin et al. 1975 [323]
20 esophageal cancer patients	819 ng/mL			quantitative real-time PCR	Herrera et al. 2005 [288]
23 patients with gastroesophageal reflux disease	432 ng/mL				
11 healthy subjects	11 ng/mL				
25 fresh samples from lung cancer patients	15 ng/mL				
38 esophageal cancer patients (fresh samples)	13 ng/mL				
28 patients with gastroesophageal reflux disease (fresh samples)	10.5 ng/mL				
46 colorectal cancer patients		stage I 560 ng/mL		spectrometry	Ito et al. 2003 [324]
7 healthy subjects		stage II 720 ng/mL			
		stage III 609 ng/mL			
		stage IV 769 ng/mL			
		320 ng/mL			
30 unselected cancer patients	219 ng/mL			competitive PCR	Jahr et al. 2001 [202]
20 healthy subjects	3.7 ng/mL				
59 healthy subjects	20 ng/mL			fluorometric method based on Sybr Green I	Jung et al. 2004 [42]
34 patients with benign prostate hyperplasia	28 ng/mL				
32 prostate cancer patients, pN0M0	20 ng/mL				
30 prostate cancer patients, pN1M0	26 ng/mL				
29 prostate cancer patients, M1	41 ng/mL				
108 healthy subjects	14 ng/mL		142 ng/mL	fluorometric method	Kamm et al. 1972 [325]

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Table 1 (continued)

Patient population	DNA in plasma	DNA in serum	RNA in plasma	Quantification method	References/remarks
99 primary breast cancer patients	0.135 absorption units			Cell death detection ELISA kit	Kuroi et al. 2001 [65]
26 recurrent breast cancer patients	0.182 absorption units				
27 patients with different types of cancer	0.244 absorption units				
11 benign breast disease patients	0.01 absorption units				
8 controls	38 ng/mL		250 ng/mL (in 6/9 healthy subjects no RNA was detectable in plasma)	2-step fluorescence assay	Laktionov et al. 2004 [43]
9 lung cancer patients	82 ng/mL		920 ng/mL		
16 patients with benign lung disease	209 ng/mL (in 3 pats. no DNA was detectable)		694 ng/mL (in one pat. no RNA was detectable)		
11 breast cancer patients	462 ng/mL		no RNA detectable in any of the patients		
58 colorectal cancer patients	105–709 ng/mL			Fluorometry	Lecomte et al. 2002 [326] in 33 patients the plasma DNA conc. was <100 ng/mL and in 25 pats >100 ng/mL
18 healthy subjects	0.1 ng/mL (in 16 subjects no plasma DNA was detectable at all)	70 ng/mL		quantitative real-time PCR	Lee et al. 2001 [280]
55 controls		13 ng/mL (42/55 samples were below detection limit, i.e. <25 ng/mL)		RIA	Leon et al. 1977 [37]
70 rheumatoid arthritis patients		116 ng/mL			
173 tumor patients ^b		180 ng/mL			
22 sex-mismatched bone marrow transplantation patients	7.7 ng/mL				
14 healthy volunteers	8.3 ng/mL			real-time PCR	Lui et al. 2001 [207]
7 sex-mismatched bone marrow transplantation patients		108 ng/mL			
45 primary lung cancer patients	mean plasma DNA conc. was significantly higher in tumor pats. than in the other groups (cut-off 80 ng/mL)			dot-blot hybridization	Maebo et al. 1990 [48] in 71% of the tumor patients and 37% of the pats. with benign diseases the DNA level was above the cut-off value,
54 patients with benign pulmonary diseases					
59 healthy subjects					
47 pancreatic cancer patients		730 ng/mL		fluorometric method based on Hoechst dye	Maire et al. 2002 [96]
31 chronic pancreatitis patients		560 ng/mL			
healthy subjects		263 ng/mL		PicoGreen quantification kit	Mori et al. 2005 [163]
47 melanoma patients		318 ng/mL (23 pats who responded to therapy) 887 ng/mL (24 pats who did not respond to therapy)			

Table 1 (continued)

Patient population	DNA in plasma	DNA in serum	RNA in plasma	Quantification method	References/remarks
20 pancreas adenocarcinoma patients	568 ng/mL			spectrophotometry	Moriyama et al. 2002 [259]
9 patients with other pancreato-biliary malignant tumors	462 ng/mL				
3 chronic pancreatitis patients	343 ng/mL				
54 archived serum samples from allergy patients		82 ng/mL		semiquantitative real-time PCR	Nagy et al. 2004 [327]
43 patients with a variety of different malignancies		24.5 µg/mL and 4.5 µg/mL in 2 ALL patients,		Diphenylamine method	Perlin et al. 1972 [328]
73 lung cancer patients			34,600 copies/mL BL	quantitative real-time PCR for GAPDH mRNA	no DNA was found in 41 patients and 31 controls Schmidt et al. 2005 [70]
56 patients with benign lung disease			9.200 copies/mL BL		BL = cell-free bronchial lavage supernatant
199 patients with malignant gastrointestinal disease		412 ng/mL		Radioimmuno-assay (RIA)	Shapiro et al. 1983 [329]
187 patients with benign gastrointestinal disease		118 ng/mL			
88 healthy subjects		14 ng/mL			
62 breast cancer patients	115 ng/mL			Spectrophotometry	Silva et al. 1999 [330]
142 breast cancer patients	135 ng/mL			Spectrophotometry	Silva et al. 2002 [331]
35 healthy subjects	15 ng/mL				
41 breast cancer patients	122 ng/mL			Spectrophotometry	Silva et al. 2002 [137]
84 NSCLC patients	318 ng/mL			DipStick Kit (Invitrogen)	Sozzi et al. 2001 [51]
32 control subjects	18 ng/mL				
100 control subjects	3 ng/mL			quantitative real-time PCR	Sozzi et al. 2003 [49]
100 NSCLC patients	24 ng/mL				
16 healthy subjects	not detectable	1.9 µg/mL		counterimmuno-electrophoresis	Steinman et al. 1975 [332]
20 healthy subjects	3–22 ng/mL			PicoGreen [®] dsDNA quantification kit	Stemmer et al. 2003 [333] the yield of plasma DNA varied with the isolation method applied
23 lung or colon cancer patients	13 ng/mL (Qiagen)				
	34 ng/mL (modified Qiagen)				
	46 ng/mL (magnetic beads)				
7 healthy subjects	960 ng/mL (after storage at 4 °C for at least 15 days)			diphenylamine staining	Stroun 1987 [8]
10 cancer with various malignancies	2.1 mg/mL				No DNA at all could be extracted from 20 mL fresh plasma from 50 healthy subjects and only after storage at 4 °C DNA could be isolated from healthy control samples
44 primary lung cancer			860 ng/mL	spectrophotometry	Sueoka et al. 2005 [196]
7 patients with solid tumors			660 ng/mL		
24 patients with benign lung disease			780 ng/mL		
25 healthy subjects			710 ng/mL		
25 healthy subjects	only 2/25 cases had free DNA, no quantity given	9–79 ng/mL		PicoGreen assay	Taback et al. 2004 [334]

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Table 1 (continued)

Patient population	DNA in plasma	DNA in serum	RNA in plasma	Quantification method	References/remarks
10 melanoma patients	259 ng/mL	1.056 ng/mL			
26 patients with liver metastasis from colorectal cancer	10.6 ng/mL	47.6 ng/mL		quantitative real-time PCR and PicoGreen dsDNA Quantitation kit (Molecular Probes)	Thijssen et al. 2002 [58]
28 healthy subjects	4.8 ng/mL	12.9 ng/mL			
41 nasopharyngeal carcinoma patients	29 ng/mL			quantitative real-time PCR	Wong et al. 2004 [335] the 43 normal subjects included 14 EBV serology positive and 29 EBV serology negative subjects
43 normal subjects	17 ng/mL				
100 healthy subjects		57 ng/mL		PicoGreen [®] dsDNA quantification kit	Wu et al. 2002 [336]
186 breast cancer patients		153 ng/mL			
74 prostate cancer patients		471 ng/mL			
161 ovarian cancer patients		85 ng/mL			
44 healthy subjects	11.6 ng/mL			PicoGreen [®] dsDNA quantification kit	Xie et al. 2004 [46]
36 patients with benign lung disease	45.5 ng/mL				
67 lung cancer patients	111 ng/mL				

observation was made for pancreatic patients during radio-chemotherapy [66]. The analysis of the nucleosome level in advanced stage NSCLC patients undergoing chemotherapy demonstrated that the nucleosome level for the area under the curve of responding patients was smaller than in non-responders [67]. A comparison between a DNA-ELISA test and a quantitative real-time PCR for the determination of cell-free DNA in plasma and serum showed that both methods are equally useful for monitoring the release of DNA into the circulation [68]. Both methods employ completely different quantification methods, leading to the conclusion, that the evaluation of nucleosome levels in combination with other tumor markers might be a valuable tool for an early estimation of the efficacy of a given therapy in cancer patients [69]. In addition to DNA quantification, the quantification of extracellular RNA is also able to discriminate between patients with a malignant lung disease and patients with a benign lung disease, since it was found that the RNA quantity in tumor patients is significantly higher than in the group of non-tumor patients [70].

Summarizing this chapter we conclude that an increased nucleic acid level in plasma/serum or any other body fluid is not specific for a defined disease. Therefore a quantitative analysis of CNAs will only have a limited value as a diagnostic marker, but in combination with other clinical parameters and/or qualitative molecular markers, it might be a useful tool for the evaluation of therapy response.

4. Alterations at the DNA level

In extracellular DNA changes such as point mutations, microsatellite alterations, single nucleotide polymorphisms (SNPs), hypermethylated sequences, rearranged genomic

sequences, and the presence of viral DNA have been detected. Additionally, mutations in mitochondrial DNA were found and the total amount of circulating DNA was determined.

The detection of genomic rearrangements and amplified sequences has only been described in a few papers. Plasma DNA from patients with B-cell malignancies were examined for tumor-associated rearranged immunoglobulin heavy-chain sequences and in 86% of the patients this clonal rearrangement was detected in the tumor DNA and in their plasma [71]. In the second paper, the detection of a translocation involving chromosomes 14 and 18 in the plasma of follicular lymphoma patients is described [72]. The detection of an amplified Her-2/neu sequence in DNA isolated from cerebrospinal fluid of patients with intracranial neoplasma was also described [73]. In addition, an amplification of the MYC-N gene in neuroblastoma patients could be demonstrated in DNA isolated from plasma or serum and preliminary data suggest that its determination might be a useful marker for follow-up of patients after therapy [74,75].

Apart from the above mentioned changes, point mutations in the mitochondrial DNA have been found in CNAs [76–81]. Since the copy number of mitochondrial DNA is much higher than that of genomic sequences, the detection of mutated sequences should be easier. Nevertheless, due to technical problems and low prevalence associated with the detection assays and clinical relevance, only a few papers have been published on this subject and on the quantification of mtDNA [82].

5. Detection of point mutations in CNAs

Mutated ras genes were the first tumor-associated alterations detected in free-circulating plasma DNA [83,84]. The most frequently gene analyzed is K-ras (Table 2, see www.cnaps.net),

Table 2
Point mutations detectable in different body fluids of cancer patients

Patient population	Gene	Tumor	Plasma/serum or other body fluids	Clinical meaning	Method	Reference/remarks
44 pancreatic adenocarcinoma patients	K-ras	28/39 (72%)	12/44 (27%)	presence of plasma mutation + survival	Enriched PCR and SSCP analysis	Castells et al. 1999 [91] 2/37 (5%) plasma samples from chronic pancreatitis patients tested positive
10 pancreatic cancer patients	K-ras		4/10 (40%)		PNA-mediated PCR clamping	Dabritz et al. 2005 [337]
15 pancreatic carcinoma patients	K-ras		11/15 (73%)		enriched PCR	Dai et al. 2003 [338] 2/10 (20%) plasma samples from chronic pancreatitis patients tested positive
41 pancreatic cancer patients	K-ras	34/50 (68%)	29/41 (71%)		enriched PCR	Dianxu et al. 2002 [100] no mutation in plasma DNA from 21 healthy subjects, 4 pats with chronic pancreatitis and 3 pats. with benign pancreatic tumor,
22 pancreatic cancer patients	K-ras		17/22 (77%)		Enriched PCR	Feng et al. 2000 [339] no mutation in plasma DNA from 20 healthy subjects
19 pancreatic carcinoma patients 25 chronic pancreatitis patients	K-ras		17/19 (89%) in PJ-Sup 15/19 (79%) in PJ-Sed 7/25 (28%) in PJ-Sup 5/25 (20%) in PJ-Sed		mutant allele specific amplification (MASA)	Ha et al. 2001 [306] PJ-Sup = cell-free pancreatic juice supernatant, PJ-Sed = pancreatic juice sediment,
47 pancreatic cancer patients	K-ras codon 12		22/47 (47%)		mutation allele specific amplification (MASA)	Maire et al. 20002 [96] 4/31 plasma DNA from chronic pancreatitis tested positive,
21 pancreatic cancer patients	K-ras	7 tissue samples tested positive	17/21 (81%)		PCR and restriction digestion	Mulcahy et al. 1998 [107] plasma DNA from 3 chronic pancreatitis patient and 5 healthy subjects tested negative
21 pancreatic cancer patients	K-ras	in 10 biopsies the plasma mutation was confirmed	17/21 (81%)		MASA PCR and enriched PCR	Mulcahy et al. 2000 [340]
3 pancreas carcinoma patients	K-ras	2/3 (67%)	3/3 (100%)		allele-specific amplification (ASA)	Sorenson et al. 1994 [83]
20 pancreatic cancer patients	K-ras		14/20 (70%)		enriched PCR	Theodor et al. 1999 [83] all 6 plasma DNA samples from patients with chronic pancreatitis tested negative
28 pancreatic cancer patients	K-ras	26/28 (93%)	9/28 (32%) before surgery, 4/28 (14%) after surgery		mutation specific mismatch ligation assay	Uemura et al. 2004 [341]

(continued on next page)

Table 2 (continued)

Patient population	Gene	Tumor	Plasma/serum or other body fluids	Clinical meaning	Method	Reference/remarks
21 pancreatic cancer patients	p53 exons 5–8, K-ras		9/21 (43%) in PJ-Sup 6/21 (29%) in PJ-Sed 2/11 (19%) in plasma 17/21 (81%) in PJ-Sup 15/21 (71%) in PJ-Sed		PCR-SSCP (p53) MASA (K-ras)	Wang et al. 2004 [308] PJ-Sup=cell-free pancreatic juice supernatant, PJ-Sed=pancreatic juice sediment, no p53 mutations found in pancreatic juice and plasma from 25 chronic pancreatitis patients, Yamada et al. 1998 [342]
21 Pancreatic Adenocarcinoma patients	K-ras	15/21 (71%)	9/15 (69%) pats. had the same mutation in the tumor tissue	presence of plasma mutation+larger tumor	Mutant allele-specific Amplification (MASA)	
29 pancreatic cancer patients	K-ras		2/29 (7%) serum samples 4/11 (36%) bile sample		enriched PCR	Zambon et al. 2000 [343] 2/4 mutations found in bile samples were from cell-free supernatant and 2 from cell sediment
14 colorectal cancer patients	K-ras	7/14 (50%)	6/14 (43%)		Mutant allele-specific Amplification (MASA)	Anker et al. 1997 [344]
16 colorectal carcinoma patients	K-ras		5/16 (31%)		enriched PCR	Borchers et al. 2002 [345]
4 ulcerative colitis patients			2/4 (50%)			disease duration in IBD patients was >15 years,
14 colorectal cancer patients	K-ras	7/14 (50%)	6/14 (43%)		Mutant allele-specific Amplification (MASA)	de Kok et al. 1997 [346]
240 patients referred to colonoscopy unit for symptomatic evaluation	p53 exon 5 (codons 175+248) APC exon 15	1 sample (codon 175) 1 patient had 5 bp deletion	3 samples (1 codon 175+2 codons 248) 1 patient had 5 bp deletion		combined amplification+restriction digestion (CARD), direct sequencing	Gocke et al. 2000 [347]
44 colorectal cancer patients	p53 K-ras	10/33 (33%) 16/44 (36%)	7/33 (21%) 3/44 (7%)		PCR and mismatch ligation assay (both genes)	Hibi et al. 1998 [282]
90 colorectal cancer patients	K-ras	31/90 (34%)	11/90 (12%)		mismatch ligation assay	Ito et al. 2002 [348] plasma from 59 patients w/o K-ras mutation tested negative
46 colorectal carcinoma patients	p53 exons 5–8	22/46 (48%)	3/46 (6%)	presence of p53 mutation in serum+stage and liver metastases	PCR and fluorescence based SSCP	Ito et al. 2003 [324]
31 patients with colorectal cancer	K-ras	6/19 (32%)	12/31 (39%)		enriched PCR	Kopreski et al. 1997 [293] all 28 plasma samples from healthy subjects tested negative
240 patients referred to colonoscopy unit for symptomatic evaluation	K-ras	35 tissue samples were positive	64/240 (27%)		enriched PCR	Kopreski et al. 2000 [99]
65 colorectal cancer patients	K-ras	22/30 (73%)	6/30 (20%)		PCR and restriction digestion (K-ras)	Lauschke et al. 2001 [349]
	APC exon 15	25/65 (38%)	20/65 (31%)		PCR-SSCP (APC)	

Table 2 (continued)

Patient population	Gene	Tumor	Plasma/serum or other body fluids	Clinical meaning	Method	Reference/remarks
58 colorectal cancer patients	K-ras codons 12+13	22/58 (38%)	10/58 (17%)	K-ras mutation and p16INK4A methylation + reduced 2-year survival	mutant allele specific amplification (MASA)	Lecomte et al. 2002 [326]
25 colorectal cancer patients	K-ras	16/25 (64%)	9/25 (36%)		TGGE	Lindfors et al. 2005 [105]
11 colorectal cancer patients	APC	7/11 (64%)	3/11 (22%)		PCR-SSCP	Ling et al. 2000 [350]
17 patients with large bowel carcinoma	p53	4/17 (24%)	5/17 (29%)		PCR-SSCP	Mayall et al. 1998 [351] none of 20 plasma DNA samples tested positive,
14 colorectal cancer patient	K-ras	7/14 (50%)	6/14 (43%)		MASA PCR and enriched PCR	Mulcahy et al. 2000 [340]
78 colorectal cancer patients	K-ras	41/78 (53%)	32/78 (41%)	postoperatively detection of mutant K-ras + disease recurrence	enriched PCR	Ryan et al. 2003 [106]
104 colorectal cancer patients	APC exon 15 K-ras p53 exon 4–8	46/104 (44%) 47/104 (45%) 38/104 (36%)	14/104 (13%) 16/104 (15%) 13/104 (12%)	detection of mutations + lymph node metastasis, stage and recurrence,	PCR-SSCP	Wang et al. 2004 [352] no mutation found in any gene in serum DNA from 50 healthy subjects,
25 patients with hepatocellular carcinoma	p53, exon 7, codon 249		10/25 (40%)		PCR and restriction digestion	Dong-Dong et al. 2003 [353] 4/20 plasma samples from cirrhosis patients and 2/30 from healthy subjects tested positive
25 hepatocellular carcinoma patients	p53, exon 7, codon 249		10/25 (40%)		PCR + restriction enzyme digestion	Huang et al. 2003 [113] plasma from 4/20 cirrhosis patients and 2/30 healthy controls tested positive
25 hepatocellular carcinoma patients	p53 exon 7, Ser-249	11/20 (55%)	6/20 (30%)		PCR and electrospray ionization mass spectrometry	Jackson et al. 2001 [112]
16 patients with hepatocellular carcinoma	p53, exon 7, codon 249		3/15 (20%)		electrospray ionization mass spectrometry	Jackson et al. 2003 [110]
158 hepatocellular carcinoma patients	p53 exon 7		28/158 (18%)		PCR and restriction digestion	Kimbi et al. 2005 [111] plasma from 1/62 controls tested positive
53 hepatocellular carcinoma patients	Ser-249 p53 (exon 7)		19/53 (36%)		PCR and restriction digestion	Kirk et al. 2000 [114] 2/13 pats. with cirrhosis and 3/53 controls tested positive
186 hepatocellular carcinoma patients	Ser-249 p53 (exon 7)		74/186 (40%)		PCR and restriction digestion	Kirk et al. 2005 [354]
98 patients with liver cirrhosis			15/98 (15%)			
29 hepatocellular carcinoma patients	p53 exon 7 codon 249	10/29 (34%),	7/29 (24%)		PCR and restriction digestion	Szymanska et al. 2004 [355] in 3 other patients additional p53 mutations were found in tissue but not in plasma
126 breast cancer patients	p53 exons 5–8	46/126 (36%)	30/126 (24%)	mutation in plasma + stage, tumor size, LN metastasis and ER status	PCR-SSCP	Di et al. 2003 [321].

(continued on next page)

Table 2 (continued)

Patient population	Gene	Tumor	Plasma/serum or other body fluids	Clinical meaning	Method	Reference/remarks
62 breast cancer patients	p53 exons 5–8	7/62 (11%)	3/62 (5%)	LOH and p53 mutation in plasma DNA+LN involvement, IDC and proliferative index	PCR-SSCP	Silva et al. 1999 [330]
142 breast cancer patients	p53, exons 5–8		10/142 (7%)		SSCP	Silva et al. 2002 [331]
41 breast cancer patients	p53 exons 5–8	7/41 (17%)	2/41 (5%)		PCR-SSCP	Silva et al. 2002a [137] in plasma samples taken 4–6 weeks after mastectomy no mutation was found, Garcia et al. 2006 Garci et al. 2006 [356]
142 breast cancer patients	p53 exons 5–8	104/142 (73%)	61/142 (43%)	presence of p53 mutation in plasma+ overall survival	PCR-SSCP and sequencing	Andriani et al. 2004 [298] in one tumor 2 different mutations were found
64 patients with non-small cell lung cancer	p53 exons 5–8	26/64 (41%)	19/64 (30%)		PCR and plaque hybridization assay	Bearzatto et al. 2002 [316]
35 lung cancer patients	K-ras	11/35 (31%)	0/31 (0%)		enriched PCR	Gonzalez et al. 2000 [357] patients with MA + p53 mutation in plasma had better survival than pats with only one alteration, Ramirez et al. 2003 [358]
35 small cell lung cancer patients	p53 exons 5–8	7/35 (20%)	13/35 (37%)	K-ras mutation+ reduced survival	PCR-SSCP	Otsuka et al. 2004 [103]
51 NSCLC patients	K-ras	9/51 (18%)	12/51 (23%)		Enriched PCR	Swisher et al. 2005 [305] peritoneal fluid tested positive in 28/30 pats. with p53 mutation, Dahse et al. 2002 [130]
27 ovarian cancer patients	p53 exons 5–8	12/27 (44%)	2/27 (7%)		PCR and fluorescence based SSCP	Dominguez et al. 2002 [359]
137 ovarian cancer patients	p53 exons 2–11	69/137 (50%)	21/137 (15%)	presence of plasma mutation+survival	PCR and/or sequencing, ligase detection reaction	Vasioukhin et al. 1994 [84]
54 bladder cancer patients	p53, exons 5–9	7/54 (13%)	2/54 (4%) plasma		Sequencing of PCR products	Coulet et al. 2000 [57]
27 bladder cancer patients	p53	1/27 (4%)	0/27		PCR-SSCP	Schlechte et al. 2004 [360] 1/14 control plasma samples tested positive for p53 mutation
10 patients with myelodysplastic syndrome or acute myelogenous leukaemia	K-ras	2/27 (7%)	3/27 (11%)		PCR-SSCP	Su et al. 2005 [361]
117 head and neck squamous cell carcinoma	N-ras		5/10 (50%)		hybridization of PCR products with mutation-specific probes	Botezatu et al. 2000 [217] urine samples were not subjected to centrifugation, i.e. they probably contained some cells
25 patients with a tumor of the digestive tract	p53		2/11 (18%)		mutant allele specific amplification (MASA)	
	p53 exons 5–8	20/23 (87%)	Before treatment 11/14 (79%)		TGGE+ sequencing	
			after treatment 7/19 (37%)			
96 gastric cancer patients	p53 exons 5–8	19/96 (20%)	5/96 (5%)		PCR and denaturing HPLC, sequencing	
8 pancreatic cancer patients	K-ras	5/7 (71%)	5/8 (62%) urine samples		enriched PCR	
7 colorectal cancer patients			4/7 (57%) urine samples			

Table 2 (continued)

Patient population	Gene	Tumor	Plasma/serum or other body fluids	Clinical meaning	Method	Reference/remarks
49 patients with solid tumor (pancreas, colon, breast, gastric)	K-ras		13/49 (26%)		PCR-RFLP	Hayashi et al. 2000 [102]
44 patients with advanced solid tumors	K-ras		5/25 (20%)		Enriched PCR	Kimura et al. 2004 [104]
15 breast cancer patients	p53 exons 5–8		2/15 (13%)		PCR-SSCP	Silva et al. 1999 [362]
10 small cell lung cancer patients			1/10 (10%)			
9 pancreatic carcinoma patients	K-ras	6/9 (67%)	8/9 (89%)		PCR-SSCP	Yamashita et al. 1998 [363] data in column 4 are from cell-free peritoneal or pleural effusions,
8 colorectal cancer patients		4/8 (50%)	2/8 (25%)			Yamashita et al. 1999 [307]
8 gastric carcinoma patients		1/8 (12%)	1/8 (12%)			
29 patients with cancer of the pancreas and biliary tract, gastric cancer and neuroendocrine tumor,	K-ras	14/29 (48%)	19/29 (65%) from cell-free pancreatic juice or bile		PCR-SSCP	
2 melanoma patients	BRAF gene, exon 15		mutations in plasma DNA were found but no details are given		PCR-SSCP	Vdovichenko et al. 2004 [364]

which was chosen as a target for several reasons; it is, next to the p53 tumor suppressor gene, the gene with one of the highest mutation frequencies found in tumors, its alteration is in some tumors an early event in malignant transformation, and there are 3 mutation hotspots in the codons 12, 13 and 61, which made the development of specific and sensitive tests possible. Nevertheless, there are some pitfalls associated with the ras mutation detection tests. It was shown that the sampling method (one vs. several samples/case) had an influence on the percentage of patients who tested positive for the presence of a K-ras mutation [85]. This observation is probably not limited to the detection of K-ras mutations, but is a general phenomenon. Several groups established a variety of PCR-based assays making it possible to enrich mutant sequences and to detect one altered K-ras sequence among 1000 to 10000 normal copies. All the methods rely upon the fact that most of the mutations occur in the 3 codons mentioned above. In the method developed first, the PCR primers are specifically designed to create a restriction site in the wild-type DNA, which is cut by a restriction enzyme before a second round of amplification, leading to an enrichment of the mutant sequence [86,87]. In another approach, a set of different mutant specific PCR primers are used which amplify the DNA only, if it contains the correct alteration [88]. In a variant of this method all K-ras sequences (i.e. wild-type and mutant) are amplified in a PCR. The amplicons are hybridized to oligonucleotides which bind mutant DNA only, but with this method the mutant DNA is not really enriched. Ahrendt et al. developed an oligonucleotide ligation assay, in which amplified K-ras sequences are incubated with two different oligonucleotides (one of which is labeled). The ligase only combines the two oligonucleotides in case of a complete hybridization and by choosing a set of different combinations of sequences the discrimination between mutant and wild-type DNA is made possible [89]. An

enrichment of mutant K-ras sequences is also described by Fischer et al. who used a subtractive hybridization column to get rid of wild-type DNA [90].

There are several reports in which K-ras mutations were found in cancer-free subjects, such as in plasma, pancreatic juice or tissue from chronic pancreatitis patients, in whom no evidence of neoplastic disease was described [91]. Even after a median follow-up of 9 months, no chronic pancreatitis patient with K-ras mutations had developed a pancreatic carcinoma. A similar observation was made by others who failed to demonstrate any relationship between the development of pancreatic neoplasm and the presence of a K-ras mutation in patients with chronic pancreatitis in a long-term follow-up [92]. Several papers described the presence of K-ras mutations not only in tumor tissue, but also in normal cells isolated from cancer patients [93]. Yakubovskaya et al. analyzed normal appearing lung tissue, lung tumor, lung metastases and sputum samples from patients with NSCLC for the presence of K-ras mutations, using a sensitive PCR method, which enriches for mutant DNA. They detected K-ras codon 12 mutations in 10% of lung tissues obtained from patients without lung diseases. The analysis of sputum samples of patients with NSCLC identified 47% to harbor a mutant ras allele, whereas 12.5% of the controls diagnosed with non-oncological lung diseases also carried this mutation [94,95]. When the frequency of K-ras mutations in plasma DNA from NSCLC patients and cancer-free subjects were compared, no difference was found [94], leading to the conclusion that the detection of K-ras mutations in plasma DNA is not a suitable marker for a tumor detection. The detection of KRAS2 mutations in circulating DNA had a low sensitivity, but a high specificity for the diagnosis of pancreatic cancer [96].

There seems to be a close association between chronic inflammation and development of a malignancy, and it has

been shown that stimulated human leukocytes release superoxide anion and hydrogen peroxide which, in the presence of iron, can be converted into the potent oxidant, hydroxyl radical ($\cdot\text{OH}$) [97]. These radicals cause DNA damage, which in turn can cause K-ras oncogene activation [98]. These data lead us to conclude that the detection of mutant K-ras is not necessarily and per se an indicator for the presence of a malignancy.

The largest study on the detection of K-ras mutations in plasma DNA was published by Kopreski et al. and included CRC patients and subjects without a malignant disease [99]. In this study, the concordance between plasma and tissue results was 83%. Some patients showed a mutant K-ras DNA in their plasma although there was no evidence of neoplasia on colonoscopy, but the presence of mutant K-ras DNA in plasma was significantly associated with risk factors for colorectal cancer.

Does the detection of point mutations in DNA isolated from plasma or other body fluids have any clinical significance? As can be seen from the results summarized in Table 2 (see www.cnaps.net), there are only a few papers in which a correlation between the presence of a point mutation and clinical parameters is described. In the study mentioned above [99] 27% of the patients with normal colonoscopy findings had a mutant K-ras gene, but so far it is not clear whether these patients have a higher risk for the development of CRC. The detection of a K-ras mutation in plasma DNA and an increase in the CA 19-9 tumor marker are independent events and upon combining both assays, the test sensitivity could be increased to 90% [100,101]. In other papers the presence of mutated plasma DNA before and after treatment was analyzed and a correlation between the disappearance of mutated DNA (found in pre-treatment plasma) in plasma DNA after therapy and a better outcome were reported [102,103]. In contrast, when NSCLC patients were analyzed for the presence of K-ras plasma DNA mutation, four of the patients turned negative for their post-treatment plasma, of whom 2 patients had stable disease and the others had a complete or partial response [104]. Lindfors et al. looked for the presence of K-ras mutations in plasma DNA in CRC patients 3 days after surgery and found about the same number of positive samples in the post-treatment samples as in pre-operative samples and observed no correlation between the presence of K-ras plasma mutation and relapse (follow-up time 6 to 52 months) [105]. In contrast, another group not only observed an association between the presence of postoperative serum mutant KRAS2 and disease recurrence in CRC patients, but showed that this marker had a better predictive value than Dukes' stage [106]. Larger prospective studies are needed to find the reasons for these contradicting results and the answer to the question as to whether there is a clinical meaning in patients who are free from clinical recurrence even if their post-surgery serum samples still contain mutant K-ras gene DNA. Interestingly, Mulcahy et al. observed a K-ras mutation in plasma DNA in patients who were diagnosed with pancreatic cancer 5 to 14 months later and did not find any alterations in patients with chronic pancreatitis or healthy subjects [107].

Another frequently analyzed gene is the p53 tumor suppressor gene whose mutations are scattered over a sequence

covering 200 codons [108] and there is some evidence that the detection of a mutation in codon 249 of the p53 gene might be a specific tumor marker (reviewed in Montesano et al. [109]. In patients with liver cirrhosis, a p53 mutation in codon 249 in plasma DNA can be found several years before a tumor has been detected [110,111]. The concordance between a p53 Ser-249 mutation in the plasma and in the tumor tissue in patients with a hepatocellular carcinoma (HCC) was found to be 55% [112]. Codon 249 mutations can be detected in plasma samples obtained from healthy controls living in Qidong, China, which is a high risk area for HCC mainly due to chronic hepatitis B virus infection and exposure to aflatoxin B1 [113]. This mutation could not be detected in plasma DNA from European patients with HCC or cirrhosis [114], which supports the assumption that (i) this specific missense mutation is an early event in hepatocarcinogenesis, (ii) that it might be a new and early diagnostic marker for the development of hepatocellular carcinoma in areas with a high risk for Hepatitis B virus (HBV) infection and/or heavy exposure to aflatoxin B1, and (iii) that the detection of this alteration in plasma DNA might be used as a marker for HCC in prevention or intervention trials in these high risk regions [113].

6. Microsatellite analysis

Microsatellites are short repetitive and highly polymorphic sequences, and the repeating units are one to six or more nucleotides long. The human genome contains several thousand microsatellites and the majority of them seem to be located in the non-coding sequence of the DNA. Microsatellite alterations (MA) can be demonstrated as an allelic imbalance (AI) or loss of heterozygosity (LOH) and as a shift after gel separation. The biological function of microsatellites is not clear, but they are frequently used as genetic markers in linkage analysis, for the analysis of the location of tumor suppressor genes, etc.

Since Chen et al. and Nawroz et al. [115,116] had demonstrated that it is possible to detect tumor-associated microsatellite alterations (MA) in plasma/serum DNA of cancer patients, there was a flood of publications corroborating these data (Table 3, see <http://www.cnaps.net>). While in the first papers patients with small cell lung cancer (SCLC) and head and neck tumor were analyzed, other groups made similar observations in patients with a tumor of the breast, melanoma, multiple myeloma, lung (NSCLC), colorectum, kidney, bladder, prostate, liver and ovary.

Several groups demonstrated that microsatellite alterations can be detected even at an early stage of the tumor, like stage I lung cancer patients [51,117], in plasma DNA of breast cancer patients who suffered from a carcinoma in situ tumor [118], and in serum DNA from early stage ovarian cancer patients [119]. In addition, the analysis of LOH in the serum DNA of patients with hepatocellular carcinoma (HCC) yielded a positive result in 5/7 patients with an AFP value less than 20 ng/mL, which is the normal range for healthy adults [120]. These and other examples demonstrate that it is possible to detect tumor-associated microsatellite alterations in plasma even at a very early stage of the disease [121,122].

Table 3

Detection of microsatellite alterations in cell-free DNA from plasma/serum and urine of cancer patients

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
20 breast cancer patients	DM-1	6/20 (30%)	3/20 (15%)		Chen et al. 1996 [115] tissue DNA from one patient with benign fibroadenoma displayed LOH, but these alterations were absent in the corresponding serum sample
	D17S1325	3/20 (15%)	0/20 (0%)		
7 patients with benign breast disease	DM-1	no alteration in any marker	no alteration in any marker		
11 breast cancer patients (plasma samples)	D17S1325	0/11	0/11		Chen et al. 1999 [118]
	D6S311	0/11	0/11		
	D7S522	1/11 (9%)	1/11 (9%)		
	D8S137	0/11	0/11		
	D9S169	1/11 (9%)	1/11 (9%)		
	D10S197	1/11 (9%)	1/11 (9%)		
	D13S260	0/11	0/11		
	D16S402	0/11	0/11		
	D16S421	0/11	0/11		
	D17S1325	1/11 (9%)	1/11 (9%)		
	D17S579	0/11	0/11		
	THRA	0/11	0/11		
	ER	1/11 (9%)	0/11		
	EABMD	0/11	0/11		
	DM	2/11 (9%)	1/11 (9%)		
	TP53	1/11 (9%)	0/11		
21 breast cancer patients (serum samples)	D8S321	7/21 (33%)	0/21		Chen et al. 1999a [118]
	D11S488	7/21 (33%)	1/21 (5%)		
	D16S398	0/21	0/21		
	D17S1325	3/21 (14%)	1/21 (5%)		
	D17S579	2/21 (9%)	0/21		
	ACTBP2	10/21 (48%)	2/21 (9%)		
	DM-1	6/21 (29%)	3/21 (14%)		
	Tp53.5	0/21	0/21		
	Tp53.6	0/21	0/21		
	Tp53.8	0/21	0/21		
17 breast carcinoma patients	UTS320	8/21 (38%)	4/21 (19%)		Mayall et al. 1999 [284]
	AR (X)	1/17 (6%)	0/17		
	ACTBP2	2/17 (12%)	2/17 (12%)		
	UT 762	0/17	2/17 (12%)		
	D14	0/17	0/17		
	DRLPA	0/17	0/17		
	FgA	1/17 (6%)	1/17 (6%)		
	15q11	0/17	0/17		
	GGAA4D07	1/17 (6%)	0/17		
	GGAA2E02	0/17	0/17		
	D18S34	0/17	0/17		Schwarzenbach et al. 2004 [129]
	D17S513	0/17	0/17		
34 breast cancer patients	D10S215		5/34 (15%)		
	D16S421		7/34 (21%)		
	D17S250		7/34 (21%)		Silva et al. 1999 [330] No alteration found in any of the markers in 13 plasma samples from 17 healthy subjects
	D17S865		0/34 (0%)		
62 breast cancer patients	D17S855	13/62 (21%)	8/62 (13%)		
	D17S654	20/62 (32%)	11/62 (18%)		
	D16S421	11/62 (18%)	8/62 (13%)		
	TH2	6/62 (10%)	6/62 (10%)		
	D10S197	9/62 (14%)	6/62 (10%)		Silva et al. 2002 [331] no alteration found in any of the markers in plasma DNA samples from 35 healthy subjects
	D9S161	10/62 (16%)	6/62 (10%)		
142 breast carcinoma patients	D17S855	104/142 (73%)	61/142 (43%)		
	D17S654	of the samples had at least one alteration in any marker	of the samples had at least one alteration in any marker		
	D16S421				
	TH2				Silva et al. 2002a [137]
	D10S197				
	D9S161				
41 breast cancer patients	D17S855	23/41 (56%) of the samples had at least one alteration in any marker	14/41 (34%) of the samples had at least one alteration in any marker		
	D17S654				
	D16S421				
	TH2				
	D10S197				
	D9S161				

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Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
56 early stage breast cancer patients	TP53 D17S855 D17S849 D16S421 D14S62 D14S51 D10S197 D8S321		12/56 (21%) in any of the markers		Taback et al. 2001 [365] No alteration found in any of the markers in plasma DNA from 30 healthy subjects
48 breast cancer patients	D1S228 D8S321 D10S197 D14S51 D14S62 D16S421 D17S849 D17S855	10 primary tumor samples were available and in all of them the LOH identified in the BM plasma were present in the primary tumor as well	3/38 (8%) 2/39 (5%) 1/26 (4%) 3/38 (8%) 4/34 (12%) 0/28 (0%) 0/31 (0%) 1/37 (3%)		Taback et al. 2003 [366] from 24 patients paired serum samples from peripheral blood was available, 3 serum samples showed an LOH and only one of them had the same alteration as in bone marrow plasma, no alteration was found in any of the markers in cell-free bone marrow plasma DNA from 5 healthy female subjects
14 breast cancer patients	24 markers located on 11 chromosome arms (1p, 1q, 3p, 7q, 9p, 11p, 11q, 16q, 17p, 17q, Xq)	all 16 tumor samples demonstrated one or more LOH,	15 LOHs were found in 12/30 plasma samples, 7/15 LOHs were located on 17p,		Wang et al. 2003 [136] plasma DNA from 5 healthy subjects tested with markers D1S549 D17S525, and D17S579 did not detect any alteration
30 lung cancer patients	GPX1 D3S1259 D13S227 p53	8/30 (27%) 7/30 (23%) 8/30 (27%) 12/30 (40%)	4/38 (10%) 3/38 (8%) 5/38 (13%) 7/38 (18%)		Allan et al. 2001 [297] plasma DNA was analyzed from 38 lung cancer patients
64 NSCLC patients	D3S1300 D3S1289 D3S1266 D3S2338 D3S1304	40/64 (62%) for all 5 markers	23/64 (50%) for all 5 markers		Andriani et al. 2004 [298]
24 lung cancer patients	D3S1293 D3S3700 D3S1283 D5S346 D9S171 D9S179 RB TP53 D17S800 D17S1818 D20S108 D20S170	11/24 (46%) 12/24 (50%) 10/24 (42%) 12/24 (50%) 7/24 (29%) 8/24 (33%) 6/24 (25%) 12/24 (50%) 4/24 (17%) 9/24 (37%) 4/24 (17%) 2/24 (8%)	8/24 (33%) 11/24 (46%) 9/24 (37%) 11/24 (46%) 8/24 (33%) 9/24 (37%) 5/24 (21%) 8/24 (33%) 4/24 (17%) 9/24 (37%) 6/24 (25%) 6/24 (25%)		Beau-Faller et al. 2003 [266]
34 lung cancer patients	D3S1293	11/34 (32%)	12/34 (35%)		Beau-Faller et al. 2003a [266] The control group included 3 patients with chronic cough, 1 tuberculosis, 7 COPD patients, 3 patients with pulmonary hypertension, 1 with cystic fibrosis, 2 with asthma and one with restrictive pulmonary disease.
20 patients with benign lung disease, in this group biopsy material was analyzed,	D3S3700 D3S1283 D5S346 D9S171 D9S179 RB TP53 D17S1818 D17S800 D20S108 D20S170	12/34 (35%) 10/34 (29%) 12/34 (35%) 7/34 (20%) 10/34 (29%) 6/34 (18%) 12/34 (35%) 9/24 (26%) 3/34 (9%) 4/34 (12%) 2/34 (6%)	13/34 (38%) 9/34 (26%) 18/34 (53%) 13/34 (38%) 14/34 (41%) 5/34 (15%) 11/34 (32%) 11/34 (32%) 5/34 (15%) 11/34 (32%) 11/34 (32%)		
20 patients with benign lung disease, in this group biopsy material was analyzed,	same marker panel as above	no alteration was found in any of the markers in material obtained at bronchoscopy	no alteration found in any of the markers		

Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
27 NSCLC patients	ACTBP-2	2/27 (7%)	3/27 (11%)		Bruhn et al. 2000 [367]
16 SCLC patients	AR	3/27 (11%)	2/27 (7%)		
	UT 762	3/27 (11%)	4/27 (15%)		
	ACTBP-2	1/16 (6%)	2/16 (12%)		
	AR	1/16 (6%)	1/16 (6%)		
	UT 762	2/16 (12%)	2/16 (12%)		
21 SCLC patients	UT762	11/21 (52%)	10/21 (48%)		Chen et al. 1996 [115]
	ACTBP2	6/21 (29%)	4/21 (19%)		
	AR	8/21 (38%)	7/21 (33%)		
11 SCLC patients	ACTBP2	8/11 (73%)	7/11 (64%)		Cuda et al. 2000 [368]
	UT 762	for all 5 markers	for all 5 markers		
	AR				
	D3S4103				
	D3S1300				
17 NSCLC patients	same marker panel as above	10/17 (59%) for all 5 markers	7/17 (41%) for all 5 markers		
35 SCLC patients	ACTBP2	4/16 (25%)	6/35 (17%)		Gonzalez et al. 2000 [357] no alteration in any of the markers found in plasma DNA from 13 healthy subjects
	UT 762	5/16 (31%)	12/35 (34%)		
	X(AR)	3/16(19%)	6/35 (17%)		
86 lung cancer patients	D3S1300		21/86 (24%)		
	D3S1560		31/86 (36%)		
	D8S201		40/86 (47%)		Khan et al. 2004 [279]
120 patients with benign lung disease	D3S1300		22/120 (18%)		
	D3S1560		27/120 (23%)		
	D8S201		28/120 (23%)		
22 NSCLC patients	D3S1038	4/22 (18%)	2/22 (9%)		
	D3S1611	6/22 (27%)	4/22 (18%)		Sanchez-Cespedes et al. 1998 [369]
	D3S1067	6/22 (27%)	5/22 (23%)		
	D3S1284	6/22 (27%)	3/22 (14%)		
42 lung cancer patients (36 NSCLC, 6 SCLC)	D9S942		1/30 (3%)		
	D9S171		0/30		
	D20S82		3/30 (10%)		Schmidt et al. 2004 [127] the data given in column 4 are from cell-free bronchial lavage supernatants
	UT5320		4/30 (13%)		
	ACTBP-2		5/30 (17%)		
	D3S1234		4/30 (13%)		
	D3S1300		4/30 (13%)		
	L17686		1/30 (3%)		Sozzi et al. 1999 [117]
87 NSCLC patients	D21S1245	49/87 (56%)	30/87 (34%)		
	D3S1300	in any of the markers	in any of the markers		
	D3S4103				
	D3S1234				
14 subjects with benign lung disease (infection, carcinoid or benign tumor)	same marker panel as above	1/14 (7%) in any of the markers	0/14 (0%) in any of the markers		
38 lung cancer patients	D3S1300	9/20 patients positive in plasma showed same alteration in the tumor	20/33 (61%) for at least one locus		Sozzi et al. 2001 [51] no alteration found in any of the markers in plasma DNA from 43 healthy subjects
	D3S1289				
	D3S1266				
	D3S2338				
	D3S1304				
49 Melanoma patients	before biochemotherapy		6/27 (22%)		Fujimoto et al. 2004 [370] patients who responded to biochemotherapy showed a significant lower allelic imbalance in their serum DNA than patients who did not respond to therapy
	D12S1657		5/34 (15%)		
	D12S393		4/38 (10%)		
	D12S1706		8/41 (19%)		
	D12S346		5/26 (19%)		
	after biochemotherapy		7/32 (22%)		
	D12S1657		5/38 (13%)		
	D12S393		7/41 (17%)		
	D12S1706				
	D12S346				

(continued on next page)

Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
40 melanoma patients	D1S214 D1S228 D3S1293 D6S264 IGFIIR D9S157 D9S161 D10S212 D10S216 D11S925	34/40 (85%) in any of the markers	23/40 (57%) in any of the markers		Fujiwara et al. 1999 [371]
76 melanoma patients	D1S214		2/7 (29%)		Fujiwara et al. 1999a [371]
stage I	D1S228		3/12 (25)		
stage II	D3S1293		16/30 (53%)		
stage III	D6S264		17/27 (63%)		
stage IV	IGFIIR D9S157 D9S161 D10S212 D10S216 D11S925				
57 melanoma patients	D1S228 D1S214 D3S1293 D6S264 D9S157 D9S161 D10S216 D11S925		32/57 (56%) in any of the markers		Taback et al. 2001 [134] no alteration detected in any of the markers in plasma DNA from 40 healthy subjects
41 stage IV melanoma patients	D1S228 D3S1293 D6S264 D9S157 D9S161 D10S212 D11S2000 D14S51 D14S62		3% 11% 8% 12% 3% 0% 5% 9% 3%		Taback et al. 2004 [135] no alteration found in any of the markers in plasma DNA from 40 healthy subjects
54 ovarian cancer patients	1p 8118 5q 1578 7q 273 8p 1085 12p 852 15q 1861 17p p53 18q 1468		at a threshold level for allelic proportion of 0.6, 50/54 patients showed an AI		Chang et al. 2002 [128] the markers were not microsatellites but SNPs, which were analyzed by digital PCR, no alteration found in any of the markers in plasma DNA from 31 control subjects
15 patients with ovarian cancer	8118	11/12 (92%) in any of the markers	3/20 (15%)		Chang et al. 2002a [133] the markers were not microsatellites but SNPs, which were analyzed by digital PCR, the samples in column 4 are from ascitic supernatants
3 pancreatic adenocarcinoma patients	486 273 2952		8/20 (40%) 9/20 (45%) 5/20 (25%)		
2 patients with colorectal cancer	311 852 1085		9/20 (45%) 5/20 (25%) 6/20 (30%)		
20 patients without any signs of malignancy	8118 486 273 2952 311 852 1085		1/20 (5%) 0/20 0/20 0/20 0/20 1/20 (5%) 0/20		Chang et al. 2002 [133] the markers were not microsatellites but SNPs, which were analyzed by digital PCR, the samples in column 4 are from ascitic supernatants

Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
38 ovarian cancer patients	D3S1029 D3S1228 D3S1300 D3S1481		29/38 (76%) in any of the markers		Zhang et al. 2002 [372]
40 transitional cell bladder cancer	D3S1285 D4S1540 D4S394 D6S419 D8S259 D8S513 D9S157 D9S167 D11S907 D11S929 D13S175 D14S288 D14S551 D17S654 D17S261 D17S786	31/40 (77%) in any of the markers	10/40 (25%) in any of the markers		Fornari et al. 2004 [139] analysis of the tissue samples from 28 control subjects (11 healthy subjects, 17 patients with benign prostatic hypertrophy) tested negative for all markers, analysis of plasma DNA from this patient population gave a positive test in 4/28 (14%) samples with any of the markers
36 bladder cancer patients	FGA.PCR2 MH34 (D4S243) 401 (D9S747) AFM186xc3 (D9S171) nm00089 (D17S695) c17m135rp (D17S654)	4/36 (11%) 7/36 (19%) 11/36 (30%) 13/36 (36%) 15/36 (42%) 14/36 (39%)	22/36 (61%) 15/36 (42%) 17/36 (47%) 15/36 (42%) 25/36 (69%) 17/36 (47%)	21/36 (58%) 15/36 (42%) 20/36 (55%) 19/36 (53%) 24/36 (67%) 23/36 (64%)	Utting et al. 2002 [131] values given in column 4 are for plasma
39 transitional cell bladder cancer patients	D5S476 D5S1720 D8S261 D8S560 D9S171 D9S925 D9S15 D13S153 D14S750 D14S61 D14S267 D17S799 D17S1306 D20S486 D20S607 D20S481 D20S480	18% 28% 31% 59% 38% 44% 44% 26% 44% 23% 28% 15% 23% 26% 23% 5% 26%	3% 10% 15% 51% 13% 8% 10% 13% 15% 3% 5% 10% 13% 8% 3% 3% 10%	10% 8% 18% 31% 21% 21% 23% 18% 15% 8% 10% 5% 5% 10% 5% 3% 5%	von Knobloch et al. 2002 [382] values for urine are from sediment (i.e. Cells) and not from cell-free supernatants
25 Renal cancer patients	D1S251 HTPO D3S1317 D3S587 D3S1560 D3S1289 D3S1286 D3S1038 D4S243 FGA CSF ACTBP2 D8S348 D8S307 D9S747 D9S242 IFN alpha D9S162 D11S488 THO vWA	25/25 (100%) positive in any of the tested markers	15/25 (60%) positive in any of the tested markers	19/25 (76%) positive in any of the tested markers	Eisenberger et al. 1999 [122] 5 patients with lesions of low malignant potential tested negative for serum DNA alterations

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Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
25 Renal cancer patients	D13S802 MJD D17S695 D17S654 D18S51 MBP D21S1245				
40 clear cell renal carcinoma patients	D3S1307 D3S1560 D3S1289 D3S1300	8/40 (20%) 9/40 (22%) 11/40 (27%) 7/40 (17%)	13/40 (32%) 12/40 (30%) 10/40 (25%) 9/40 (22%)		Goessl et al. 1998 [374]
53 renal cell carcinoma patients	D3S1560 D3S2450 D5S1720 D7S1797 D7S1807 D9S925 D13S153 D17S799 D17S1306	68% 57% 49% 23% 26% 26% 32% 24% 26%	6% 24% 21% 19% 17% 6% 17% 11% 17%		von Knobloch et al. 2002 [373] in 3/20 controls PCR artifacts resembling an allelic imbalance were found
117 head and neck squamous cell carcinoma patients	UT5085	65/117 (55%)	3/117 (3%)		Coulet et al. 2000 [57]
21 Head and neck cancer patients	IFNA D9S200 D9S161 D9S156 D3S1284 D3S1238 D14S50 D21S1245 CHRNA1 D17S786 FCA DRPLA		1/21 (5%) 3/21 (14%) 4/21 (19%) 2/21 (9%) 2/21 (9%) 6/21 (29%) 2/21 (9%) 8/21 (38%) 2/21 (9%) 0/21 (0%) 0/21 (0%) 2/21 (9%)		Nawroz et al. 1996 [375]
152 Head and neck squamous carcinoma patients	D9S161 D9S200 D3S1238 D3S1284 ChMB1 D14S50 Li7686 CSFIR	68/152 (45%) tested positive in any of the markers	68/152 (45%) tested positive in any of the markers		Nawroz-Danish et al. 2004 [116] association between disease-free survival and MA in serum DNA
91 patients with head and neck squamous cell carcinoma	D2S123 D13S308E D5S1501 D1S3721 D12S1052 D17S974 D17S1294 D13S800	58/91 (64%) in any of the markers	21/91 (23%) in any of the markers		Nunes et al. 2001 [376]
26 colorectal cancer patients	D1S197 D1S207 D1S225 D1S305 D2S138 D2S159 D3S1283 D3S1282 D4S394 D4S414 D5S430 D5S346 D6S275	9/26 (35%) 15/26 (58%) 14/26 (54%) 13/26 (50%) 9/26 (35%) 7/26 (27%) 4/26 (15%) 7/26 (27%) 9/26 (35%) 11/26 (42%) 5/26 (19%) 6/26 (23%) 13/26 (50%) 9/26 (35%)	11/26 (42%) 13/26 (50%) 15/26 (58%) 12/26 (46%) 8/26 (31%) 6/26 (23%) 7/26 (27%) 9/26 (35%) 11/26 (42%) 10/26 (38%) 6/26 (23%) 14/26 (54%) 10/26 (38%)		Beau-Faller et al. 2003 [266] the samples analyzed in column four are from paired colon/liver metastasis (CLM) or liver biopsy and NOT from plasma

Table 3 (continued)

Patient population	Microsatellite markers	Tumor	Plasma/serum	Urine	Reference/remarks
26 colorectal cancer patients	D6S264	8/26 (31%)	11/26 (42%)		
	D8S264	19/26 (73%)	18/26 (69%)		
	D8S283	20/26 (77%)	22/26 (85%)		
	D9S171	10/26 (38%)	12/26 (46%)		
	D9S179	6/24 (25%)	5/24 (21%)		
	D10S191	11/26 (42%)	9/26 (35%)		
	D10S192	9/26 (35%)	11/26 (42%)		
	D11S916	7/26 (27%)	12/26 (46%)		
	D13S173	13/26 (50%)	11/26 (42%)		
	D14S65	11/26 (42%)	14/26 (54%)		
	D15S127	11/26 (42%)	11/26 (42%)		
	D16S408	2/26 (8%)	3/26 (11%)		
	D16S422	8/26 (31%)	10/26 (38%)		
	TP53	22/26 (85%)	22/26 (85%)		
	D17S790	14/26 (54%)	15/26 (58%)		
	D17S794	10/26 (38%)	10/26 (38%)		
	D18S53	12/26 (46%)	15/26 (58%)		
	D18S61	20/26 (77%)	20/26 (77%)		
	D20S107	13/26 (50%)	15/26 (58%)		
	D22S928	8/26 (31%)	8/26 (31%)		
44 colorectal cancer patients	D18S55	LOH: 31/44 (70%)	LOH: 0/31		Hibi et al. 1998 [282]
	D18S58	MSI: 15/44 (34%)	MSI: 0/15		
	D18S61				
	D18S69				
	CHRNA1				
	D17S786				
	D8S254				
27 colorectal cancer patients	D8S133				
	D1S243	4/27 (15%)	1/27 (4%)		Kolble et al. 1999 [268] no alterations were found in plasma DNA from 10 healthy subjects using a panel of 6 markers
	D5S82	10/27 (37%)	6/27 (22%)		
	D8S264	16/27 (59%)	5/27 (18%)		
	D12S1689	7/27 (26%)	4/27 (15%)		
	D15S127	11/27 (41%)	5/27 (18%)		
	D15S129	5/27 (18%)	1/27 (4%)		
	D17S796	15/27 (55%)	4/27 (15%)		
	D17S1813	13/27 (48%)	8/27 (30%)		
	D18S70	14/27 (52%)	3/27 (11%)		
81 patients with multiple myeloma	D13S319		16/62 (26%)		Ahmed et al. 2003 [377]
	D13AFMaw301wb5		for both D13 markers		
	D11S2179		7/61 (11%)		
21 hepatocellular carcinoma patients	74 markers from 17 chromosomal arms	21/21 (100%) in any of the markers	16/21 (76%) in any of the markers		Chang et al. 2002 [120]
6 cholangiocarcinoma patients	35 markers from 16 chromosomal arms	5/6 (83%) in any of the markers	0/6 (0%) in any of the markers		Chang et al. 2002 [120]

The sensitivity of tests for the detection of MA is only 0.5%, but the rate of false positive results (at least for normal DNA) seems to be low [123]. Beau-Faller et al. used a panel of 12 microsatellite markers located on 9 different chromosomal regions for the analysis of lung cancer patients and found an alteration in 88% of the plasma samples from patients, while all control samples were negative for MAs [56]. Nevertheless there are reports in which MA were found in the bronchial epithelium of current and former smokers [124], in the mucosa [125] and plasma [126] of ulcerative colitis patients and in DNA from cell-free bronchial lavage supernatants of patients with a benign lung disease [127]. The clinical meaning of these alterations which are found in cells exposed to stress as in patients with long-term chronic diseases is not clear.

In theory, the combination of the analysis for LOH and a test for one or more tissue-specific serologic biomarkers might be helpful in establishing a diagnostic test which is specific for a certain type of tumor [128], but the simultaneous analysis of plasma LOH and CA15-3 in breast cancer patients demonstrated no correlation between these values [129]. A similar finding was made in a small group of hepatocellular carcinoma patients, who demonstrated an LOH in their serum DNA but showed normal AFP values, which led to the conclusion that this assay might be useful only for the discrimination of HCC from cholangiocarcinoma [120].

MA were also detected in cell-free urine [130,131] and in cell-free bronchial lavage (BL) supernatants from patients with lung cancer [127]. Interestingly, there were less alterations found in

BL cells than in cell-free BL supernatants. These results compare favourably to other reports [131,132] and are seen as an enrichment of tumor-derived DNA in plasma in comparison to cellular DNA. The use of single nucleotide polymorphisms (SNP) instead of microsatellites for the detection of allelic imbalances demonstrated a higher frequency of AI in ascitic fluid supernatant than in the cell pellets, suggesting that tumor DNA is enriched in the ascitic supernatant also [133].

There are a few papers in which LOH in plasma DNA was correlated with a variety of clinical data. Hoon and colleagues were among the first to demonstrate that the presence of MA in the plasma of melanoma patients not only has a prognostic significance [134], but that there is an association between MA in plasma and the response to biochemotherapy in these patients [135]. In other papers conflicting results were published, reflecting the fact that our basic knowledge on the biology of circulating nucleic acids is still very limited. While one group did not find any clear-cut clinical differences when breast cancer patients after therapy were examined for the presence of LOH in their plasma DNA [136], others described a correlation between the presence of molecular plasma DNA alterations and clinicopathological factors [116,137], but in the majority of papers published a clinical meaning could not be established (Table 3, see www.cnaps.net). Given the observation that it is possible to isolate intact circulating tumor cells from peripheral blood of breast cancer patients for more than two decades after therapy [138], it is no surprise that persistent molecular alterations in plasma DNA are also detectable. Whether there is an association between the presence of MA and disease stage is also not clear so far. Fornari and colleagues demonstrated that in transitional cell cancer of the bladder, the number of plasma DNA alterations were higher in patients with low grade or low stage tumors than in tumors with higher grade or stage [139]. In a follow-up study of the same group of renal cancer patients who were previously analyzed for the presence of MA in urine and serum [122], a statistically significant correlation between the frequency of MA in serum (but not in urine) and disease recurrence was observed [140].

Therefore the main challenge for future studies is to establish a panel of microsatellite markers, which, in combination with other markers, is not only specific for tumor-associated alterations but specific for a distinct tumor type and has a clinical meaning as well.

7. Methylation analysis

DNA methylation is an important epigenetic modification of the genome and involved in the regulation of many cellular processes. It is a physiological process active in all healthy cells, but a growing number of human diseases have also been found to be associated with aberrant DNA methylation (for a summary see Table 4 at www.cnaps.net) [132]. An epigenetic DNA modification, like a hypermethylation of a promoter region, results in a gene silencing without changing its coding sequence. Interrupted and/or de-regulated metabolic pathways in cancer cells are frequently associated with an aberrant CpG island methylation leading to the shut down of genes involved

in the regulation of the cell cycle, growth, and genes involved in genome stability and apoptosis induction. Assays for the detection of promoter CpG island hypermethylation have several advantages: the abnormal DNA methylation represents a stable tumor-associated marker that can be easily detected, the acquired methylation of a specific gene will generally not be lost, and compared with point mutations, which often occur at several sites within a given gene, DNA methylation occurs mainly at specific CpG islands [141]. Herman et al. developed a method, called methylation-specific PCR (MSP), which can rapidly assess the methylation status of virtually any group of CpG sites within a CpG island [142]. The isolated DNA is modified by sodium bisulfite treatment, converting unmethylated (but not methylated) cytosines to uracil and PCR amplified with primers specific for methylated or unmethylated DNA. The method was originally developed as a qualitative approach and was later improved into a quantitative assay by using fluorescence labeled probes and both methods show a high sensitivity and specificity [142,143].

An aberrant methylation of the tumor suppressor gene p16INK4a is an early event in lung cancer and a potential biomarker for early diagnosis [144], and therefore it was obvious to use MSP for the characterization of free-circulating DNA from tumor patients (summarized in Table 4, see www.cnaps.net). A hypermethylation of several genes was also detected in early stage breast cancer patients [145] and in ovarian cancer patients, in whom an aberrant methylation was found in well-differentiated stage IA/B tumors and borderline neoplasms of low malignant potential [146]. While these observations fostered the hope that it might be possible to detect cancer at an early stage, another publication showed, that there was no difference in the hypermethylation of the DAP-kinase promoter sequence in early and late stage patients with nasopharyngeal carcinoma [147].

The rate of false positive results obtained with this method seems to be very low and the introduction of quantitative assays made it possible to distinguish false positive samples from true positive ones by the degree of methylation [148]. In two papers, the frequency of plasma DNA methylation was compared with DNA isolated from white blood cells and it was consistently found, that the quantity of hypermethylated DNA was much less in the cellular DNA [149,150], suggesting that there is an enrichment of tumor DNA in the plasma (an observation which was made for other genetic alterations, like LOH, as well, see above). When the quantity of methylated DNA in plasma and serum was compared, it was found that methylated APC-DNA was detectable in 93% of the plasma samples but only 40% of the serum samples, suggesting that larger quantities of tumor DNA are present in plasma [151]. In contrast to these results there was no difference in the quantity of methylated p16INK4A DNA from plasma and serum isolated from HCC patients [152]. Whether these conflicting observations are due to the different methods applied (qualitative assays vs. a quantitative approach) is so far unknown. When the quantity of methylated APC 1A promoter DNA in lung cancer patients was examined, a wide range of methylated APC detectable in tumor tissues was found [151]. This variation was attributed to the

Table 4
Presence of methylated DNA sequences in CNAs

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
34 Breast cancer patients	APC RASSF1A DAP-kinase	15/34 (44%) 22/34 (65%) 17/34 (50%)	10/34 (29%) 19/34 (56%) 12/34 (35%)			MSP		Dulaimi et al. 2004 [145] no methylation in any of the genes in plasma DNA from 20 healthy controls and 8 pats with benign breast disease
148 Breast cancer patients	RASSF1A		29/148 (20%)			quantitative real-time PCR	persistence of RASSF1A plasma methylation 1 year after therapy + poor outcome	Fiegl et al. 2005 [378] 11/154 (7%) patients with benign breast disease were positive in serum DNA
36 Breast cancer patients	P16INK4A CDH1	4/36 (11%) 9/36 (25%)	3/36 (8%) 7/36 (19%)			MSP	plasma DNA methylation + nodal metastasis	Hu et al. 2003 [379]
50 Breast cancer patients	ESR 1 APC HSD17B4 HIC1		7/26 (27%) 6/26 (23%) 3/26 (12%) 10/26 (39%)			quantitative real-time PCR	methylation of RASSF1A and/or APC + poor outcome	Muller et al. 2003 [269] plasma results are for the 26 patients from the gene evaluation set only,
Breast cancer patients	RASSF1A RARβ APC CDH1 CDH13		8/17 (47%) 5/15 (33%) 4/10 (40%) 4/9 (44%) 3/8 (37%)			MSP		Rykova et al. 2004 [143] 1/6 plasma samples from pats. with benign breast disease were positive for CDH1 and CDH13 methylation
43 Breast cancer patients	p16INK4A	10/43 (23%)	6/43 (14%)			PCR + restriction enzyme digestion	Presence of MA, p53 mutation and p16 methylation in plasma DNA + involvement of >3 LN, high proliferative index and IDC	Silva et al. 1999 [380]
41 Breast cancer patients	p16INK4a	9/41 (22%)	4/41 (10%)			MSP		Silva et al. 2002 [137] 2/4 patients retained plasma DNA methylation after mastectomy
50 Ovarian or primary peritoneal tumor patients	BRCA1 RASSF1A APC p14 p16 DAPK	12/50 (24%) 25/50 (50%) 5/5 (100%) 3/3 (100%) 5/5 (100%) 3/3 (100%)	9/50 (18%) 20/50 (40%) 3/5 (60%) 3/3 (100%) 5/5 (100%) 3/3 (100%)	7/43 (16%) 12/43 (28%) 4/5 (80%) 1/3 (33%) 4/4 (100%) 2/3 (67%)		MSP		Ibanez et al. 2004 [146] no methylation in any of the genes in cyst tissue, serum, or peritoneal fluid DNA from 10 patients with benign ovarian disease, in serum DNA from 20 normal subjects and in 10 nonneoplastic ovarian tissue DNA
57 Ovarian cancer patients	TIMP 3 CDH1 CDH13 APC PPP1R13B HSPA2 HSD17B4 ESR1 GSTP1			29/57 (51%) 48/57 (84%) 34/57 (60%) 34/57 (60%) 22/57 (39%) 29/57 (51%) 20/57 (35%) 44/57 (77%) 8/57 (14%)		MSP	fewer methylated genes + shorter overall survival	Muller et al. 2004 [381]

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Table 4 (continued)

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
57 Ovarian cancer patients	CYP1B BRCA1 MYOD1 SOCS1 TITF1 GSTM3			18/57 (32%) 25/57 (44%) 46/57 (81%) 31/57 (54%) 21/57 (37%) 26/57 (46%)				
41 Gastric cancer patients	p16 E-cadherin RARβ		9/41 (22%) 9/41 (22%) 6/41 (15%)			MSP		Koike et al. 2004 [382] No aberrant methylation in serum from 10 volunteers
44 Primary gastric cancer patients	p16INK4A E-cadherin	24/44 (54%) 23/44 (52%)	16/44 (36%) positive in one or both genes			MSP		Ichikawa et al. 2004 [383] no methylation in serum from 10 healthy subjects
60 Gastric carcinoma patients	p16INK4a	23/60 (38%)	6/60 (10%)			MSP		Kanyama et al. 2003 [384]
54 Gastric adenocarcinoma patients	DAPK E-cadherin GSTP1 p15 p16INK4A	38/54 (70%) 41/54 (76%) 10/54 (18%) 37/54 (68%) 36/54 (67%)	26/54 (48%) 31/54 (57%) 8/54 (15%) 30/54 (55%) 28/54 (52%)			MSP		Lee et al. 2002 [385]
26 Renal clear cell carcinoma	APC ARF CDH1 GSTP1 MGMT p16 RAR-β2, RASSF1A TIMP3	5/17 (29%) 4/17 (25%) 10/17 (59%) 2/17 (12%) 1/17 (6%) 6/17 (35%) 9/17 (53%) 15/17 (88%) 12/17 (71%)	1/18 (6%) 1/18 (6%) 6/18 (33%) 1/18 (6%) 0/18 (0%) 4/18 (22%) 1/18 (6%) 2/18 (11%) 3/18 (17%)		10/26 (38%) 8/26 (31%) 10/26 (38%) 4/26 (15%) 2/26 (8%) 9/26 (35%) 8/26 (31%) 17/26 (65%) 12/26 (46%) the results with urine are from sediment, i.e. cells	quantitative real-time PCR		Hoque et al. 2004 [148] no methylation found in urine DNA from 91 control subjects without history of genitourinary neoplasm in four genes examined (p16, MGMT, GSTP1, ARF)no promoter hypermethylation in four genes (p16, RAR-β2, TIMP3, GSTP1) in serum DNA of 30 controls
46 Hepatocellular carcinoma patients	p16INK4A		22/46 (48%)			MSP		Chu et al. 2004 [160] false positives in plasma DNA from 4/23 pats with liver cirrhosis
22 Hepatocellular carcinoma patients	p16INK4a	16/22 (73%)	13/22 (59%)			MSP		Wong et al. 1999 [150] no false positives in serum of non-HCC patients,
25 Hepatocellular carcinoma patients	P15 P16INK4A	16/25 (64%) 19/25 (76%)	4/25 (16%) 15/25 (60%)			MSP	p15 and p16 methylation + development of metastasis/recurrence	Wong et al. 2000 [386] no false positives in plasma DNA from 35 healthy controls and pats. with chronic hepatitis for p15 methylation,
45 Hepatocellular carcinoma patients	p16INK4A	30/45 (67%)	24/45 (53%)			MSP	methylation in plasma + preoperative serum AFP level	Wong et al. 2000a [161] no false positives in plasma DNA from 35 non-HCC patients
45 Hepatocellular carcinoma patients	p16INK4A	30/45 (67%)	14/45 (31%)			quantitative real-time PCR	decrease of methylation index after surgery	Wong et al. 2003 [152] no false positives in 50 plasma samples from non-HCC patients,
105 Lung cancer patients	p16INK4A	73/92 (79%)	77/105 (73%)			MSP		An et al. 2000 [387]

Table 4 (continued)

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
35 NSCLC patients	p16INK4A	20/35 (57%)	2/20 (10%)			MSP		Bearzatto et al. 2002 [316] no false positives in plasma DNA from 15 healthy subjects and 15 pats. with an unmethylated tumor,
56 Stage I NSCLC survivors	p16INK4A MGMT RASSF1A		11/44 (25%) 5 (11%) 3 (7%)	10/53 (19%) 19/53 (36%) 13/53 (24%)		nested MSP	methylation of multiple gene promoters in sputum + lung cancer risk in comparison to cancer-free smokers	Belinsky et al. 2005 [267] data in column 5 are from sputum samples,
121 Smokers (40 pack years)	p16INK4A MGMT RASSF1A		16/121 (13%) 15/121 (12%) 2/121 (2%)	30/118 (25%) 17/118 (14%) 8/118 (7%)				
74 Never smokers	p16INK4A MGMT RASSF1A		7/74 (9%) 2/74 (3%) 2/74 (3%)					
22 Non-small cell lung cancer patients	p16INK4A DAPK GSTP1 MGMT	9/22 (41%) 5/22 (22%) 2/22 (9%) 6/22 (27%)	3/22 (14%) 4/22 (18%) 1/22 (5%) 4/22 (18%)			MSP		Esteller et al. 1999 [162] no false positives in 22 paired normal lung tissues in any marker
91 Lung cancer patient	MGMT p16INK4A RASSF1A DAPKinase RARβ	23/30 (77%) tissue samples were methylated in at least one gene,	17/91 (12%) 14/91 (15%) 11/91 (12%) 10/91 (11%) 6/91 (7%)				methylation of 2 or more genes + diagnosis of lung cancer	
100 Patients with benign lung disease	MGMT p16INK4A RASSF1A DAPKinase RARβ		2/100 (2%) 3/100 (3%) 8/100 (8%) 5/100 (5%) 1/100 (1%)			MSP		Fujiwara et al. 2005 [388]
33 NSCLC patients	p16INK4A	14/33 (42%)	2/14 (14%)			MSP		Ng et al. 2002 [389]
50 Lung cancer patients	RASSF1A DAPK TMS1	17/50 (34%) 23/50 (46%) 18/50 (36%)	17/50 (34%) 20/50 (40%) 17/50 (34%)			MSP		Ramirez et al. 2003 [358]
115 NSCLC patients	14-3-3σ		39/115 (34%)			MSP	methylation and survival	Ramirez et al. 2005 [164]
9 Lung cancer patients	RASSF1A APC		4/9 (44%) 3/9 (33%)			MSP		Rykova et al. 2004 [143] no false positives in plasma DNA from 16 pats with benign lung diseases
99 Lung cancer patients	APC	95/99 (96%)	42/89 (47%)			quantitative real-time PCR		Usadel et al. 2002 [151] no false positives in plasma of patients without methylation of tumor DNA and 50 healthy controls,
46 Lung cancer patients	DAPKinase MGMT p16INK4A RARβ2 RASSF1A FHIT p14ARF APC E-cadherin APC 1B		0/36 1/43 (2%) 5/45 (11%) 19/46 (41%) 2/44 (4%) 18/44 (41%) 15/45 (33%) 3/46 (6%) 25/32 (78%) 2/35 (6%)			MSP		Zemaitis et al. 2005 [390] no false positives in plasma DNA from 14 healthy subjects in markers DAPKinase, MGMT, p16INK4A, FHIT, APC 1A
19 Colorectal cancer patients	hMLH1	9/19 (47%)	3/19 (16%)			MSP		Grady et al. 2001 [391]

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Table 4 (continued)

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
58 Colorectal cancer patients	p16INK4a	31/58 (53%)	21/58 (36%)			MSP	presence of K-ras mutation and p16INK4A methylation in plasma DNA + shortened disease free survival	Lecomte et al. 2002 [326]
94 Colorectal cancer patients	p16INK4A	44/94 (47%)	13/94 (14%)			MSP		Nakayama et al. 2002 [392]
11 Recurrent colorectal cancer	P16INK4A	8/11 (73%)	7/11 (64%)			MSP		Nakayama et al. 2003 [393]
34 Recurrent colorectal cancer	P16INK4A		24/34 (71%)					Plasma DNA from 50 CRC pats. without p16 tumor methylation were negative, Yamaguchi et al. 2003 [394]
122 Colorectal cancer patients	DAPK	67/122 (55%)	3/18 (17%)			MSP		Zou et al. 2002 [395]
52 Colorectal cancer patients	p16INK4A	20/52 (38%)	14/52 (27%)			MSP	p16INK4A methylation + advanced stage	no false positives in serum DNA from 34 pats. with polyps and 10 healthy controls, Goessl et al. 2000 [396]
32 Prostate cancer patients	GSTP1	16/17 (94%)	23/32 (72%)	4/8 (50%) in ejaculate	4/11 (36%) in urine sediment	fluorescent MSP		no false positives in tissues, plasma and ejaculates from 15 BPH patients
69 Prostate adenocarcinoma patients	GSTP1	63/69 (91%)	9/69 (13%)		13/69 (19%) in urine sediment	MSP/quantitative real-time PCR		Jeronimo et al. 2002 [397] 9/31 BPH tissue samples were positive but all 31 plasma samples were negative
7 Prostate cancer patients	CD44		7/7 (100%)			MSP		Vis et al. 2001 [398]
95 Head and neck cancer patients	P16INK4A MGMT DAPK GSTP1	26/95 (27%) 31/95 (33%) 17/92 (18%) 0/41	8/26 (31%) 14/29 (48%) 3/17 (18%) no data			MSP	DAPK methylation + lymph node involvement and advanced stage	Sanchez-Cespedes et al. 2000 [399]
20 Head and neck squamous cell carcinoma patients	p16INK4A p15		13/20 (65%), mean conc. 56 copies/mL 12/20 (60%), mean conc. 65 copies/mL			quantitative real-time PCR		Wong et al. 2003 [153] of 24 healthy subjects 4/20 (20%) tested positive for p16INK4A and 12/24 (50%) for p15
27 Bladder cancer patients	P14ARF p16INK4A	15/27 (55%) 5/27 (18%)	13/27 (48%) 2/27 (7%)				p14ARF methylation + larger tumors + relapse	Dominguez et al. 2002 [359]
86 Bladder cancer patients	p16INK4A	14/53 (26%)	19/86 (22%)			MSP		Valenzuela et al. 2002 [400] no false positives in serum DNA from controls and pats. without tumor DNA methylation
38 Esophageal squamous cell carcinoma patients	p16INK4A	31/38 (82%)	7/38 (18%)			MSP		Hibi et al. 2001 [401]

Table 4 (continued)

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
32 Esophageal squamous cell carcinoma patients	APC	16/32 (50%)	2/32 (6%)			quantitative real-time PCR	APC methylation+ advanced stage and reduced survival	Kawakami et al. 2000 [402] no false positives in tissue and plasma DNA from 20 patients with normal esophageal epithelium
52 Esophageal adenocarcinoma patients	APC	48/52 (92%)	13/52 (25%)					
43 Nondysplastic Barrett's esophagus patients	APC	17/43 (39%)	0/11					
37 Normal gastric mucosa samples	APC	15/37 (40%)	0/23 patients with gastritis					
32 Nasopharyngeal carcinoma patients	DAPK	24/32 (75%)	5/12 (42%)			MSP		Wong et al. 2002 [147] no false positives found in normal nasopharyngeal tissue from 5 patients
41 Nasopharyngeal carcinoma patients	CDH1 DAPKinase p15 p16INK4a RASSF1A MLH1		19/41 (46%) 8/41 (19%) 8/41 (19%) 17/41 (41%) 2/41 (5%) 0/41					Wong et al. 2004 [335] no methylated plasma DNA was found in 43 normal subjects
93 Cervical cancer patients	CALCA hTERT MYOD1 PGR TIMP3	19/19 (100%) 6/19 (32%) 19/19 (100%) 19/19 (100%) 1/19 (5%)	62% 0% 25% 73/93 (78%) 4%			quantitative real-time PCR	unmethylated MYOD1+ better survival, MYOD1 methylation+ higher stage	Widschwendter et al. 2004 [166]
93 Cervical cancer patients	CDH 1 CDH 13		39/93 (42%) 4/93 (4%)			quantitative real-time PCR	unmethylated plasma DNA+ better disease free survival, higher methylation frequency in both genes+ enhanced relapse rate,	Widschwendter et al. 2004 [403]
37 Lymphoma patients	p16INK4A		27/37 (73%)			methylation-sensitive restriction enzyme-related PCR		Deligezer et al. 2003 [149] no false positives in plasma from 20 healthy subjects,
28 Glioblastoma multiforme patients	MGMT p16INK4A DAPK RASSF1A	8/21 (38%) 14/21 (67%) 11/21 (52%) 12/21 (57%)	11/28 (39%) 15/27 (55%) 9/26 (35%) 13/26 (50%)			MSP	MGMT methylation+ BCNU treatment response	Balana et al. 2003 [404]
50 Melanoma patients before therapy	RASSF1A RAR-β2 MGMT		13/50 (26%) 10/50 (20%) 5/50 (10%)			MSP	methylation of RASSF1A and response to biochemotherapy	Mori et al. 2005 [163] all serum samples from healthy subjects tested negative for all 3 genes
38 adult AML patients	P15	23/38 (60%)	Methylation found in 9 adult AML and 7 adult ALL patients			MSP	P15 methylation and reduced survival	Wong et al. 2000 [405]
13 adult ALL patients		9/13 (69%)						
4 AML children		4/4 (100%)						
21 ALL children		8/21 (38%)						
38 adult AML patients		7/30 (23%)						

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Table 4 (continued)

Patient population	Genes analyzed	Positive in the tumor	Positive in plasma/serum	Positive in peritoneal fluid/washing, ascites fluid	Positive in urine	Detection method	Clinical association	Reference/remarks
13 adult ALL patients	P16INK4A	1/8 (12%)						
4 AML children		0/2 (0%)						
21 ALL children		0/8 (0%)						

heterogeneity of tumors harboring cells with either methylated or unmethylated APC, as well as to differences in the stromal cell contamination of the tumors. The determination of the fractional concentration of circulating tumor-derived DNA, measured as methylated p16INK4A DNA, which was isolated from plasma/serum of HCC patients, yielded a variation from 0.2 to 100% [152], confirming work published before [151].

Since no single genetic alteration can be seen as absolutely tumor specific, a qualitative detection of any alteration cannot be viewed as proof for the presence of a tumor, and a quantitative approach should be added. This was also demonstrated in the analysis of methylated p15 and p16INK4A plasma DNA from 24 healthy subjects, in which 20% of the subjects tested positive for p16INK4A and 50% for p15 plasma DNA methylation, but the mean concentration of methylated sequences for both genes was significantly lower in the control group than in the tumor patients [153]. As mentioned above, the hypermethylation of regulatory sequences is a physiological process present in every normal cell, a fact which has to be taken into consideration, when the methylation of tumor cell DNA is analyzed. Müller et al. examined serum DNA from breast cancer patients and healthy women in early stage pregnancy and compared the methylation status of six genes i.e. TIMP-3, CDH-1, PTHS-2, BLT1, APC and RASSF1A [154]. They found that the methylation frequency of the genes, known to be involved in the process of tumor invasion (CDH-1, TIMP-3 and PTHS-2) and immune response (BLT1) were at about the same level in both groups. The similarity in the methylation patterns of genes known to be involved in metastasis and tumor cell invasion, in the invasion process of trophoblast cells, and in the regulation of the immune response, in pregnant women and metastasized breast cancer patients clearly demonstrates, that cells which show a different methylation pattern, do not automatically have to be of tumor origin. To make things even more complicated, methylation changes are age-dependent and before any attempts are made using DNA methylation for an early detection of tumors, the methylation patterns in normal tissues have to be thoroughly analyzed. The observation that a hypermethylation of the GSTP1 promoter sequence is not only found in prostatic adenocarcinoma [155] but also in prostatic intraepithelial neoplasia, [156] makes a differentiation of these patient groups based solely on this marker impossible. In addition, we examined the methylation status of several genes (RASSF1A and APC) in plasma DNA from patients with a histologically confirmed lung cancer and from patients with benign lung

disease and detected an aberrant methylation in both patient groups [157]. The detection of an aberrant methylation pattern of several genes in sputum from cancer free patients was also described [158].

The number of analyzed samples might have an impact on the results of an experiment as was demonstrated by Wong et al., who looked for an aberrant p16INK4a methylation in the DNA from plasma and serum of 22 liver cancer patients and detected no association with any clinical data [150]. Upon extending their analysis and incorporating 23 additional patients into the same study, they found a significant association between the presence of p16INK4a methylation and the development of tumor recurrence or metastases [159]. Other groups also combined data from the analysis of methylated plasma DNA and tumor markers and yielded conflicting results. While in one paper no association between the methylation of free-circulating p16INK4A serum DNA and AFP concentration was found [160], others observed a significant correlation between these variables [161]. The methylation of the APC gene in serum samples of lung cancer patients was found to be an independent prognostic factor [151], but no correlation between the methylation status of the genes coding for p16INK4a, DAPK, GSTP1, and MGMT in serum samples of lung cancer patients and prognosis was found [162]. The detection of aberrantly methylated APC DNA in plasma from lung cancer patients might have a prognostic value [151]. The detection of methylated RASSF1A gene in plasma DNA of melanoma patients and its correlation with survival and response to biochemotherapy [163], and the association between methylation of the 14-3-3sigma gene and survival of NSCLC patients [164] demonstrate that these analyses might be clinically meaningful. Thus the choice of appropriate target genes is one of the main factors to attain clinical significance [165,166].

8. Detection of viral sequences

Even before free-circulating nucleic acids became popular as a target for tumor marker search, the presence of cell-free viral nucleic acids had been described [167]. The strong association of a virus infection and the development of certain types of cancer makes the detection of viral nucleic acids an interesting field (Table 5, see www.cnaps.net).

There are several lines of evidence that the plasma Epstein–Barr virus DNA is derived from the tumor; the viral DNA is detectable in the majority of NPC patients, but at a much lower

Table 5

Overview on the detection of virus nucleic acid in plasma/serum and other body fluids in tumor patients

Patient population	Virus/target sequence	Tumor	Plasma serum or other body fluids	Clinical meaning	Method	Reference/remarks
170 nasopharyngeal carcinoma patients	EBV, <i>Bam</i> H1-W		median pretreatment EBV DNA level 2352 copies/mL, median post-treatment EBV DNA 0 copies/mL	higher level of post-treatment EBV DNA + overall survival	real-time quantitative PCR	Chan et al. 2002 [180]
36 nasopharyngeal carcinoma patients	EBV, EBER-1		27/36 (75%)		qualitative PCR	Hsiao et al. 2002 [414] 3/28 (11%) serum samples of control subjects tested positive
29 nasopharyngeal carcinoma patients	EBV, EBNA-1	20/29 (69%)	15/29 (52%)		qualitative PCR	Krishna et al. 2004 [415] 6/26 (23%) tissue and 2/12 (17%) serum samples from pats with nasal polyps tested positive
58 nasopharyngeal carcinoma patients	EBV, pol-1 <i>Bam</i> H1-W <i>Lmp</i> 2		0–6,600 copies in pre-treatment sera (pol-1 assay),	post-treatment EBV DNA level + freedom from relapse	real-time quantitative PCR	Le et al. 2005 [416]
90 nasopharyngeal carcinoma patients with stage I and II	EBV, <i>Bam</i> H1-W			high pretreatment EBV DNA level (cutoff 4000 copies/mL) + distant relapse	real-time quantitative PCR	Leung et al. 2003 [417]
24 nasopharyngeal carcinoma patients with local recurrence	EBV, <i>Bam</i> H1-W		15/24 (63%), median EBV DNA level 24 copies/mL, 24/25 (96%)		real-time quantitative PCR	Leung et al. 2003 [417] the plasma EBV DNA level in newly diagnosed patients are much higher than in pats with local recurrence (similar stages were compared)
25 nasopharyngeal carcinoma patients with distant metastasis						
139 nasopharyngeal carcinoma patients	EBV, <i>Bam</i> H1-W		132/139 (95%)	combination of EBV DNA and IgA-VCA test gives 99% diagnostic sensitivity	real-time quantitative PCR	Leung et al. 2004 [169]
99 nasopharyngeal carcinoma patients	EBV, <i>Bam</i> H1-W		stage III (25 pats): 681 copies/mL stage IV (74 pats): 1703 copies/mL, M1 (19 pats): 291,940 copies/mL	pre-treatment EBV DNA level (cutoff 1500 copies/mL) + overall and relapse-free survival, post-treatment EBV DNA level and overall and relapse-free survival,	real-time quantitative PCR	Lin et al. 2004 [176] plasma DNA from 40 healthy volunteers and 20 patients cured from NPC tested all negative,
26 nasopharyngeal carcinoma patients	EBV, EBER-1 RNA		23/26 (88%)		qualitative RT-PCR and hybridization	Lo et al. 1999 [185] 6/29 (21%) plasma samples from healthy subjects tested positive
10 nasopharyngeal carcinoma patients with recurrence	EBV, <i>Bam</i> H1-W		median EBV DNA 32,350 copies/mL	16/17 patients had a reduction of EBV DNA after radiotherapy, development of recurrence + increased EBV DNA level,	real-time quantitative PCR	Lo et al. 1999 [418]

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Table 5 (continued)

Patient population	Virus/target sequence	Tumor	Plasma serum or other body fluids	Clinical meaning	Method	Reference/remarks
15 nasopharyngeal carcinoma patients in remission	EBV,		median EBV DNA 0 copies/mL	increased plasma EBV DNA level+stage	real-time quantitative PCR	Lo et al. 1999 [147] median EBV DNA in 43 healthy subjects was 0 copies/mL (3/43 samples tested positive)
57 nasopharyngeal carcinoma patients			median EBV DNA 21,058 copies/mL			
91 nasopharyngeal carcinoma patients (19 pats. with recurrence, 72 pats. without recurrence in the first year)	BamH1-W, EBNA-1		median EBV DNA level in 19 pats: 41,756 copies/mL, median level in 72 pats: 5,807 copies/mL	increased plasma EBV DNA level+early relapse, serum EBV DNA level+NPC-related death (139 pats. with long follow-up)	real-time quantitative PCR	Lo et al. 2000 [419]
139 nasopharyngeal carcinoma patients with follow-up for >5 years	EBV, BamH1-W		102/107 (95%)	median EBV DNA level in plasma+tumor stage	real-time quantitative PCR	Lo et al. 2000 [420]
107 nasopharyngeal carcinoma patients					real-time quantitative PCR	
6 patients with locally advanced, nonmetastatic nasopharyngeal carcinoma	EBV, BamH1-W			4/6 patients with normal PET scans and reduction of EBV DNA level were clinically free of recurrent disease, 2 pats had elevated EBV DNA level after therapy and subsequently relapsed,	real-time quantitative PCR	Makitie et al. 2004 [421]
42 nasopharyngeal carcinoma patients	EBV, EBNA-2	42/42 (100%)	13/42 (31%)		qualitative PCR	Mutirangura et al. 1998 [422] sera from 82 seropositive healthy subjects tested negative
150 nasopharyngeal carcinoma patients	EBV, EBNA-1	49/49 (100%)	138/150 (92%)		real-time quantitative PCR	Shao et al. 2004 [423] 9/75 (12%) plasma samples from healthy subjects tested positive
167 nasopharyngeal carcinoma patients	EBV, EBNA-2		98/167 (59%)		qualitative PCR	Shotelersuk et al. 2000 [33] 10/77 (13%) plasma samples from healthy subjects tested positive
28 patients with early recurrent nasopharyngeal carcinoma	EBV, BamH1-W		mean 255 copies/mL before surgery	complete surgical tumor removal+decrease of EBV DNA on day 7 after surgery	real-time quantitative PCR	Wei et al. 2004 [424]
101 patients with nonnasopharyngeal head and neck carcinomas	EBV, BamH1-W		mean 30 copies/mL after surgery	plasma EBV DNA conc. is a reflection of the tumor EBER status	real-time quantitative PCR	Yu et al. 2004 [168]
48 healthy controls			3827 copies/mL in pats with EBER-positive tumors			

Table 5 (continued)

Patient population	Virus/target sequence	Tumor	Plasma serum or other body fluids	Clinical meaning	Method	Reference/remarks
39 EBV-positive lymphoma patients	EBV, EBNA-1		median EBV DNA in 21 NK cell lymphomas at presentation 6.1×10^7 copies/mL, in 7 patients with CR the EBV level became undetectable, in 14 refractory or relapsing cases the titer remained high,	EBV DNA level at presentation for the whole group of EBV-positive lymphoma patients+ overall survival and disease-free survival, decrease of EBV DNA during treatment+ overall survival	real-time quantitative PCR	Au et al. 2004 [425] plasma from 34 EBV negative lymphomas tested negative
5 patients with lymphoproliferative disease after allogeneic bone marrow or peripheral blood stem cell transplantation	EBV, EBNA-1		cell-free EBV DNA was detected in all patients during PTLT, in 4/5 pats. presence of EBV DNA preceded onset of PTLT,		qualitative PCR	Beck et al. 1999 [426] 8/96 plasma samples from 41 BMT or PBST pats. without clinical signs of PTLT tested positive,
30 Hodgkin's disease patients	EBV, BamH1-W		all 30 samples tested positive (range 250 – 2.5×10^7 copies/mL)		PCR-ELISA	Drouet et al. 1999 [427]
33 EBV-associated Hodgkin's disease (HD) patients	EBV, BamH1-W		30/33 (91%)		qualitative PCR and hybridization	Gallagher et al. 1999 [35] all 15 serum samples from healthy subjects tested negative
26 non-EBV associated HD patients			6/26 (23%)			
12 infectious mononucleosis patients			5/12 (42%)			
37 newly diagnosed Hodgkin disease patients	BALF5 gene of EBV		all 8 EBV-positive pats. tested positive before therapy, all 21 pats. in remission tested negative	serial monitoring could predict therapy response	real-time quantitative PCR	Gandhi et al. 2006 [428]
21 HD patients in long-term remission						
13 patients with EBV-associated lymphoid malignancies	EBV, BamH1-W	12/13 (92%)	11/11 (100%) median EBV DNA level 2266 copies/mL		real-time quantitative PCR	Lei et al. 2000 [172] all 35 plasma samples from healthy subjects tested negative,
18 patients with NK/T-cell lymphoma	EBV, BamH1-W		17/18 (94%), median EBV level 659 copies/mL	decrease of plasma EBV DNA level+ therapy response, high plasma EBV DNA level before therapy+ poor survival in 3 prospectively analyzed patients with longitudinal serum analysis there was a correlation between clinical response to therapy+ EBER-1 DNA	real-time quantitative PCR	Lei et al. 2002 [429] all 35 plasma samples from control subjects tested negative,
6 patients with EBV-associated posttransplant lymphoproliferative disease (PTLD)	EBV, EBER-1		5/6 (83%) at the time of diagnosis of PTLT		PCR and hybridization	Limaye et al. 1999 [430] all 16 serum samples from transplant patients without PTLT tested negative,

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Table 5 (continued)

Patient population	Virus/target sequence	Tumor	Plasma serum or other body fluids	Clinical meaning	Method	Reference/remarks
5 patients with lymphoepithelioma-like carcinoma of the lung	EBV, EBER-1		median EBV DNA level 9613 copies/mL		real-time quantitative PCR	Ngan et al. 2002 [431] lack of correlation between IgAVCA and EBV DNA in plasma Clin Cancer
19 patients with lymphoepithelioma-like carcinoma of the lung	EBV, EBER-1		mean 75,340 copies/mL	pre-treatment EBV DNA level + survival (cutoff 10,000 copies/mL)	real-time quantitative PCR	Ngan et al. 2004 [179]
14 patients with EBV-associated lymphoproliferative disease (LPD)	EBV, BNRF1 p143		14/14 pats. had elevated EBV DNA levels at EBV-LPD diagnosis		real-time quantitative PCR	van Esser et al. 2001 [173] plasma samples from healthy EBV-positive individuals tested negative
28 paediatric Hodgkin's disease pats. before treatment,	EBV, BamH1-W		13/24 (54%) pats. before treatment tested positive,		real-time quantitative PCR	Wagner et al. 2001 [432]
12 paediatric HD patients after treatment			pats. in stable remission after treatment tested all negative			
14 HPV positive cervical adenocarcinoma patient	HPV-16 E7 or HPV-18 E7		12/14 (86%)		real-time quantitative PCR	Dong et al. 2002 [433] all 28 plasma samples from pats. with a negative qualitative PCR result tested negative in the quantitative assay also
40 cervical cancer patients	HPV-16 E6/E7		7/40 (18%)		qualitative PCR	Liu et al. 2001 [434]
20 HPV-16 positive cervical cancer patients	HPV-18 E6/E7 HPV-16 E6/E7		1/40 (2%) 3/20 (15%)			
63 cervical cancer patients	HPV-E6		6/50 (12%)		qualitative PCR and Southern hybridization	Pornthanakasem et al. 2001 [435]
94 cervical cancer patients	HPV L1 open reading frame	85/94 (90%)	42/94 (45%)	post-treatment HPV detection in plasma and recurrence	qualitative PCR	Widschwendter et al. 2003 [436]
50 cervical cancer patients	HPV16 HPV18	13/18 (72%)	25/50 (50%) cancer pats. 6/18 (33%) with high grade lesions.		real-time qualitative PCR	Yang et al. 2004 [170]
18 patients with high-grade cervical lesions		4/18 (22%)	3/15 (20%) with low grade lesions.			
15 patients with low-grade cervical lesions			3/50 (6%) cancer pats.			
41 patients with acute infectious mononucleosis (IM)	EBV, large internal repeat region		11/41 (27%)		qualitative PCR and Southern hybridization	Gan et al. 1994 [437] all 18 sera from patients with nasopharyngeal carcinoma tested negative
20 patients with infectious mononucleosis (IM)	EBV, BamH1-W		13/13 acute phase IM 4/9 convalescent phase IM		qualitative PCR	Yamamoto et al. 1995 [438] sera from 20 EBV seropositive and 18 EBV seronegative children tested negative

Table 5 (continued)

Patient population	Virus/target sequence	Tumor	Plasma serum or other body fluids	Clinical meaning	Method	Reference/remarks
18 seropositive children with infectious mononucleosis (IM)	EBV, <i>Bam</i> H1-W		14/18 (78%) 138 to 17,620 copies/mL		qualitative PCR and DNA enzyme immunoassay	Zingg et al. 1999 [32] all samples from 30 blood donors (29 positive for IgG to VCA) tested negative
9 children with EBV-associated tumors 9 children without-EBV-associated tumors	EBV, <i>Bam</i> H1-W		mean EBV DNA level 34,229 copies/mL no EBV DNA detectable	EBV DNA levels in sequential specimens from children with infectious mononucleosis or EBV-associated lymphomas correlated with disease activity,	real-time quantitative PCR	Berger et al. 2001 [439]
72 head and neck squamous cell carcinoma patients	HPV-16, L1 region	15/51 (29%)	6 plasma samples tested positive		PCR and dot blot hybridization	Capone et al. 2000 [440]
19 patients with chronic active nonfatal EBV infection 14 patients with chronic active fatal EBV infection	EBV, <i>Bam</i> H1-W		2/19 (10%) 9/14 (64%)		qualitative PCR	Kanegane et al. 1999 [441] plasma from 2/18 (11%) patients with acute EBV-induced infections mononucleosis tested positive, all 10 plasma samples from healthy subjects tested negative
51 gastric carcinoma patients	EBV, <i>Bam</i> H1-W		5/5 plasma from EBER positive pats. tested positive for EBV DNA		real-time quantitative PCR	Lo et al. 2001 [442] all plasma samples from 32 EBER-negative patients tested negative for EBV DNA, serum from 7/30 gastritis patients and 7/197 healthy subjects tested positive for EVB DNA but with much lower copy number than EBER positive tumor patients, Wagner et al. 2001 [443] plasma from 20 immunocompetent children tested negative
96 randomly selected renal transplanted children and adolescents	EBV, <i>Bam</i> H1-W and <i>Bam</i> H1-K		meand EBV DNA level in pats with posttransplant lymphoproliferative disorder 32,250 copies/mL	EBV was more effectively cleared from plasma than from WBC, viral load 10,000 copie/mL was 100% specific and sensitive for PTL	real-time quantitative PCR	

rate in control populations, and the copy number of viral DNA is usually higher in the tumor patient population than in the controls, although there is an overlap. When patients with non-nasopharyngeal head and neck carcinomas (NNHNC) were examined, plasma EBV DNA concentrations in patients with EBV-encoding RNA-positive (EBER) tumors were significantly higher than those in the controls [168]. The comparison of circulating EBV DNA and anti-EBV capsid antigen IgA (IgA VCA) showed that there is a detectable level of EBV DNA and IgA-VCA in 2–4% of healthy individuals without cancer [169],

but it is not known whether this group of healthy subjects carries an increased risk for the development of a tumor. The same is true for the small but consistent control population who tested positive for plasma EBV DNA and for plasma HPV16/HPV18-DNA [170].

In contrast to other tumor-associated genetic markers detectable in plasma, the presence of cell-free viral nucleic acids, especially EBV, seems to have a strong clinical impact, as there is a good correlation between the plasma EBV DNA level and the disease activity and a serial examination of

plasma DNA might be a valuable tool for therapy monitoring [171]. Patients who responded to a therapy demonstrated a significant reduction of plasma EBV DNA, whereas there was no change in non-responders [172,173]. There is a close association between the presence and copy number of EBV DNA in plasma and the disappearance of EBV DNA from the plasma of NPC patients showing a complete response to a therapy [174]. The detection of an elevation of EBV plasma DNA in NPC patients with a local or distant relapse was possible up to 6 months before the recurrence was detected clinically [175,176]. In addition to viral DNA, the detection of HBV RNA in human serum has been shown to have a diagnostic potential for the discrimination of apparent and inapparent stages of HBV infection [177].

Is the pre-treatment or post-treatment determination of the EBV DNA level in plasma clinically more meaningful? In two studies it was shown that the pre-therapy EBV DNA level has a prognostic value and that a sustained clearance of serum EBV DNA correlates with therapy response and prolonged tumor control [178,179]. In contrast, other observations showed that the levels of post-treatment EBV DNA dominated the effect of pre-treatment EBV DNA for both progression-free survival and overall survival [180].

To be useful as a genetic marker, the half-life of plasma EBV DNA has to be short. Lo et al. followed the decay rate in NPC patients treated with radiation therapy and determined this rate to be 3.8 days [181], but they postulated that this rate might rather be a reflection of dying tumor cells than the actual half-life of EBV DNA. The monitoring of the decay of the DNA in patients undergoing surgical removal of their tumor showed a median half-life of plasma EBV DNA of 139 min [182] and this rapid elimination of circulating EBV DNA makes it an ideal marker to be used for EBV-associated diseases.

Taken together, the aforementioned data point to the fact, that the measurement of free-circulating EBV DNA is not only useful in a diagnostic setting, but might be a reliable tool for the monitoring of patients, for the assessment of a given therapy and for prognostic purposes as well.

9. Detection of expressed/overexpressed RNA in CNAs

RNA is much more fragile than DNA and prone to degradation by the ubiquitous presence of RNA degrading enzymes [183]. Since it is known that the serum RNase concentration is elevated in cancer patients [184], it was quite a surprise when Lo et al. found EBV-associated RNA in the plasma of nasopharyngeal carcinoma patients [185] and Kopreski et al. published their observations on the detection of free-circulating mRNA isolated from serum of melanoma patients [186]. The examination of mRNA from 6 patients with malignant melanoma for the presence of tyrosinase mRNA, a gene which is overexpressed in melanoma cells, demonstrated a positive result in 4 of them. Since then, the detection of over/expressed tumor-associated genes has been described in patients with cancer of the lung, thyroid, breast, prostate, bladder, and melanoma. (Table 6, see www.cnaps.net). RNA molecules were also found in human milk in a concentration of 20 to 70 µg/mL

[187]. The majority of these RNA molecules had a size of 100 bp and less, down to single nucleotides. A few fragments which were cloned and sequenced correspond to ribosomal RNA, different tRNAs and 28s and 18 sRNA.

Published reports upon the association between the detection of free-circulating plasma mRNA and clinical data yielded contradictory results. Wieczorek et al. were the first to suggest that the RNA–proteolipid complex found in sera of tumor patients had a unique chemical composition and a half-life of approximately 2 days [188]. In addition, they observed a correlation between the level of circulating RNA and the tumor mass and proposed that the RNA might be a clinically useful marker. Silva et al. found an association between the detection of free-circulating mRNA coding for mammaglobin and CK19 genes in plasma and clinicopathological parameters (tumor size and proliferative index) in breast cancer patients [189], and in CRC patients the β -catenin mRNA concentration in plasma was associated with tumor stage [190]. A positive correlation was also found between the presence of CEA and CK 19 plasma RNA and advanced stage of CRC patients and the presence of circulating tumor cells in these patients [191]. Others demonstrated the possibility to monitor disease and to detect a cancer in tumor patients using a real-time quantification of hTERT plasma mRNA [192]. Interestingly, Wong et al. observed a decrease of plasma β -catenin mRNA concentration in 16/19 colorectal cancer patients after successful surgery [190]. In contrast, several studies did not find a correlation between the detection of telomerase subunits encoding mRNA in the serum or plasma of breast cancer patients and clinical data (i.e. tumor size, grade and presence of lymph node metastases) [193–195]. Also, Sueoka et al. did not find a correlation between hnRNP B1 mRNA levels and clinical parameters (such as stage, white blood cell count, or C-reactive protein level in the peripheral blood) in lung cancer patients [196].

Therefore, the question whether a quantitative analysis of free-circulating RNA could be useful for diagnostic purposes remains to be answered, but it seems feasible that such an assay might be a useful clinical tool.

10. The ins and outs of CNAs — where do they come from, how long do they persist and how are they removed?

Several groups performed experiments to find out how CNAs are released — by active secretion or apoptosis or necrosis. One study showed that the amount of Alu repeat sequences in plasma/serum DNA is increased compared to lymphocyte DNA and that an active release of DNA is an important factor in the origin of CNAs [197], but apoptosis is another mechanism also contributing to extracellular DNA [198]. Actively growing (in vitro) normal and malignant cells are able to shed nucleic acids into the cultivation medium, while dead cells did not perform in these experiments [199,200]. When in vitro cultivated cells were treated to initiate apoptosis, an increase of released DNA was observed, while heat treated cells (necrosis initiation) were unable to produce an increase in the quantity of extracellular DNA [199,200]. Plasma DNA isolated from hemodialysis patients and systemic lupus erythematosus (SLE) patients showed a ladder-

Table 6
Detection of tumor-associated overexpressed cell-free mRNA in plasma/serum of cancer patients

Patient population	Genes	Plasma/serum	Method	Reference/remarks
18 breast cancer patients	hTR hTERT	5/18 (28%) 4/16 (25%)	qualitative RT-PCR	Chen et al. 2000 [193] plasma samples from 2 patients with benign breast disease and 21 healthy subjects tested all negative in both genes
10 breast cancer patients	mammaglobin	6/10 (60%)	qualitative RT-PCR	Gal et al. 2001 [195] 6/16 (38%) plasma samples from healthy subjects tested positive
45 breast cancer patients	mammaglobin CK 19	27/45 (60%) 22/45 (49%)	qualitative RT-PCR	Silva et al. 2001 [189]
25 healthy subjects	mammaglobin CK 19	3/25 (12%) 5/25 (20%)		
8 lung cancer patients before any therapy	Her2/neu hnRNP-B1	4/8 (50%) 8/8 (100%)	qualitative RT-PCR	Fleischhacker et al. 2001 [406] serum RNA
10 lung cancer patients after/during chemotherapy	Her2/neu hnRNP-B1	3/10 (33%) 6/10 (60%)		samples from 12 healthy subjects tested all negative in both markers
25 lung cancer patients (cell-free bronchial lavage supernatant was analyzed but not plasma)	hnRNP-B1 PGP 9.5 MAGE-2 Her2/neu Aurora Pericentrin hTR hTERT	18/25 (72%) 8/25 (32%) 20/25 (80%) 6/25 (24%) 4/25 (16%) 12/25 (48%) 25/25 (100%) 3/25 (12%)	qualitative RT-PCR	Schmidt et al. 2004 [127]
44 lung cancer patients	hnRNP B1	0.99pg/g RNA	quantitative real-time PCR	Sueoka et al. 2005 [196] overexpression of the hnRNP B1 gene was also detected in plasma samples from 7 patients with other solid tumors,
24 patients with benign lung disease	hnRNP B1	0.3pg/g RNA		Lledo et al. 2004 [194]
25 healthy subjects	hnRNP B1	0.23pg/g RNA		
50 colorectal cancer patients	hTERT	detectable in both groups, but significantly higher expressed in tumor samples	quantitative real-time PCR	
50 healthy subjects				
53 colorectal cancer patients	CEA CK 19	17/53 (32%) 39/53 (74%)	nested RT-PCR	Silva et al. 2002 [191]
25 healthy subjects	CEA CK 19	1/25 (4%) 5/25 (20%)		
58 colorectal cancer patients	β -catenin	58/58 (100%)	quantitative real-time PCR	Wong et al. 2004 [190]
49 colorectal adenoma patients	β -catenin	49/49 (100%)		
43 healthy subjects	β -catenin	36/43 (84%)		
10 malignant melanoma patients	Tyrosinase gp100 MART-1 PBGD	3/10 (30%) in serum and 5/10 (50%) in plasma 0/10 in plasma and serum 0/10 in plasma and serum 7/10 (70%) in serum and 8/10 (80%) in plasma	qualitative RT-PCR	Hasselmann et al. 2001 [407]
119 melanoma patients	tyrosinase MAGE-3 MUC-18 p97	24/119 (20%) 10/119 (8%) 41/119 (34%) 19/119 (16%)	RT-PCR Southern blot	Hoon et al. 2000 [408]
6 melanoma patients	Tyrosinase c-abl	4/6 (67%) 6/6 (100%)	Nested RT-PCR	Kopreski et al. 1999 [186]
20 healthy subjects	Tyrosinase c-abl	0/20 (0%) 20/20 (100%)		
9 follicular lymphoma patients	hTERT	detectable in all 3 groups, but significantly higher expressed in tumor samples,	quantitative real-time PCR	Dasi et al. 2001 [192]
9 colorectal cancer patients				
10 healthy subjects				
5 breast cancer patients	5T4	2/5 (40%)	seminested RT-PCR	Kopreski et al. 2001 [409]
14 lung cancer patients		6/14 (43%)		
46 healthy subjects		3/25 (12%)		
25 breast cancer patients	hTR hTERT	23/25 (92%) 12/25 (48%)	qualitative RT-PCR	Novakovic et al. 2004 [410]

(continued on next page)

Table 6 (continued)

Patient population	Genes	Plasma/serum	Method	Reference/remarks
29 malignant melanoma patients	hTR	24/29 (83%)		
	hTERT	17/29 (59%)		
4 thyroid cancer patients	hTR	4/4 (100%)		
	hTERT	4/4 (100%)		
7 healthy subjects	hTR	3/7 (43%)		
	hTERT	0/7 (0%)		
78 hepatocellular carcinoma	hTERT	90%	Qualitative	Miura et al. 2003 [411]
10 liver cirrhosis patients		70%	RT-PCR	
12 chronic hepatitis patients		42%		
64 hepatocellular carcinoma patients	hTERT	the hTERT mRNA level in HCC patients was significantly higher than in patients with liver cirrhosis and chronic hepatitis	quantitative real-time PCR	Miura et al. 2005 [412]
20 patients with liver cirrhosis				
20 chronic hepatitis patients				
12 prostatic carcinoma patients before therapy	PSMA	4/12 (33%)	qualitative	Papadopoulou et al. 2004 [413]
	CEA	1/12 (8%)	RT-PCR	
19 prostate cancer patients during therapy	PSMA	3/19 (16%)		
	CEA	3/19 (16%)		
18 healthy subjects	PSMA	0/9 (0%)		
	CEA	negative in all tested subjects		

like pattern characteristic for apoptotic degradation [201]. In contrast, Jahr et al. induced apoptosis and necrosis of in vitro cultivated cells and demonstrated (i) an increase of released DNA after either treatment, (ii) a “DNA ladder” which is typical for apoptosis and (iii) a high molecular weight DNA from cells in which necrosis was induced [202]. In other reports, dying hematopoietic cells were made responsible for the release of cell-free DNA into the circulation, since it had been observed that patients undergoing hemodialysis showed an increased level of plasma DNA [203,204,201]. When the plasma DNA isolated from hemodialysis patients and SLE patients were compared, both samples showed the same ladder-like pattern when analyzed on PAGE gels, which led the authors to conclude that in both patient groups the oligonucleosome-like structures, released into the plasma, are due to apoptosis [201]. That macrophages play an important role in the release of extracellular DNA was demonstrated when these cells were co-cultivated with apoptotic or necrotic cells. An increase of released DNA into the medium in a dose-dependent manner was seen when necrotic cells were used, while the coculture of apoptotic cells with macrophages caused a decrease in the amount of released DNA [205,206].

To determine the origin of plasma DNA, female and male patients who received bone marrow or an organ transplant from a donor of the opposite sex were analyzed and it was demonstrated that the majority of circulating DNA was from the hematopoietic system [207,208].

The half-life of CNAs seems to be very short as was shown when purified DNA was injected into the blood stream of animals [209–212]. Viral DNA, which is a closed ring, survives much longer than bacterial DNA which is a linear molecule [213]. Experiments in mice showed that DNA/anti-DNA immune complexes were rapidly cleared from the circulation by the liver [214]. The half-life of DNA isolated from hemodialysis patients was determined to be approximately 4 min [201] and the half-life of circulating fetal DNA is approximately 16 min [215]. The clearance mechanism is not known, since the

extracellular fetal DNA was not secreted in the urine [216]. In contrast, radiolabeled bacteriophage lambda DNA, subcutaneously injected into mice, can be isolated from the urine of the animals within 3 days after injection. Y-specific DNA sequences can also be detected in the urine of women receiving a blood transfusion from a male donor or being pregnant with a male fetus [217]. In some cases it was also possible to detect mutant K-ras gene sequences in the urine of patients with pancreatic or colorectal cancer, demonstrating that at least part of the extracellular DNA is excreted with the urine [218]. A different path for the removal of extracellular DNA is by liver uptake, as 70% to 85% of radiolabeled mono-nucleosomes were removed from the circulation of mice within 10 min [219].

11. Do CNAs have a biological meaning?

There are several lines of evidence pointing to a biological function of circulating nucleic acids. The traffic of RNA plays an important role in cell–cell communication and development in plants [220] and it has recently been demonstrated, that the mRNA coding for a gene which induces flowering migrates from the leave to the shoot apex [221]. Another example is the development of crown galls which are considered a plant tumor, even if the existence of a “true” malignancy in the plant kingdom is questioned [222,223]. The primary gall is the result of a genetic transformation of plant cells by bacteria (*Agrobacterium tumefaciens*) which deliver the so-called transfer DNA (T-DNA) [224]. This T-DNA eventually integrates into the plant cell genome and takes over part of the cellular machinery, resulting in the cancerous phenotype. The foreign DNA can also move freely from cell to cell in the plant, where it enters nuclei of other cells. Its information is transcribed and expressed, leading to the formation of secondary tumors, i.e. metastases [7]. Since plant cells are fixed to their position and not able to move around as in animals, the “transforming principle”, i.e. the T-DNA, must be circulating.

That DNA is an amazingly robust molecule and resistant against degradation was demonstrated in mice in whom orally

ingested DNA not only survives the digestion in the gastrointestinal tract, but appears in the blood cells and the serum [225] and is even detectable in the fetuses of pregnant mice [226]. Virtually all cultured animal cells (including human cells) are able to import almost any presented DNA in a very fast and efficient manner [6,227]. The ability to import DNA-containing complexes [199,228] and RNA-containing vesicles [24,30] was described in several papers. Evidence for a cell–cell information transport based on a transfer of RNA was presented by Jachertz [229] and by Anker et al., who showed that DNA isolated from the supernatant of virus-exposed T cells is able to trigger the synthesis of virus directed antibodies in B lymphocytes [230]. Also, the production of specific antibodies expressing human characteristics was demonstrated after nude mice had been injected with DNA released by antigen stimulated human T lymphocytes [231] and the binding of purified DNA or nucleosomes to murine spleen cells resulted in a release of large amounts of interleukin-6 [232].

The so-called I-somes, originally found in the cytoplasm of embryonic muscle tissue, contain a special class of cytoplasmic DNA, i.e. informational DNA, which may act as a carrier of genetic information from the nucleus to the cytoplasm, and can also be extruded [233]. These I-somes are associated with RNA and contain a complete system for information transfer. In vitro cultivated mouse spleen lymphocytes show an increase in ³H-thymidine incorporation when they are incubated with tumor-cell conditioned growth medium [234]. When tumor cells are incubated in a medium containing cytosol from normal cells, the ³H-thymidine incorporation rate is reduced, indicating (i) that there is a competition between these DNA complexes from normal and tumor cells, and (ii) that these extruded DNA complexes may be “transmitters of genetic information.”

The secretion and transfer of macromolecular RNA between mammalian cells has been described as well [235,236]. Based on the observations that a variety of different RNases have a cytostatic effect, and that a tumor angiogenesis factor has a sequence 40% identical to human RNase and is catalytically active as an RNase, Benner developed the hypothesis that a combination of RNA, RNases and RNase inhibitors control the tissue development in higher organisms [237]. An RNA proteolipid complex, consisting of a 27S RNA and proteolipid-bound glycosphingolipids and isolated from the serum of melanoblastoma patients, can transform reticulum cells from bone marrow [238]. After addition of this RNA–proteolipid complex to the culture medium a dramatic change in the morphology of the cells, including a polymorphism of cell shape, and a rise in the proliferation rate was observed. RNA from plasmocytoma cells from tumor-bearing mice is able to convert lymphocyte antigen receptors of B cells in vitro and in vivo and this RNA has a suppressive effect on the primary immune response [239,240]. In humans the ability of transmitting information for the synthesis of surface immunoglobulins between myeloma cells and normal lymphocytes was also demonstrated [241]. Such an RNA complex may represent a specific secretory product of the tumor cells, able to mediate host–tumor interaction [19] or mediates the communication between eukaryotic genes and their gene products [242]. HeLa

cells are able to incorporate exogenously added mononucleotides, and larger oligonucleotide segments into newly synthesized RNA [243]. RNA isolated from Ehrlich carcinoma ascites fluid is able to elicit a specific stimulating action on the transplantability and growth of a homologous tumor [244,245]. This RNA is formed as a result of its release from intact tumor cells, and seems to be one of the factors responsible for the interaction between the tumor and the organism.

So far the mechanism/s which are responsible for a horizontal gene transfer are not clear, but apoptosis, i.e. programmed cell death, might be one factor. The formation of apoptotic bodies, which contain either DNA or RNA [246] is increased in tumor patients, especially during and after therapy. de la Taille et al. established a cocultivation system and demonstrated a transfer of genetic material between two human prostate cancer cell lines [247]. In another approach, Holmgren et al. showed that apoptotic particles contain different genes which can be transferred to and expressed in recipient cells [248,249].

Several researchers asked the question whether an in vivo transfection of normal cells with CNAs might lead to the development of a tumor. According to a theoretical calculation, the answer to this question is yes [250]. The possibility to transform murine cells with DNA isolated from transformed cells, which led to the discovery of dominant oncogenes, seems to be a confirmation of this statement [251]. Nevertheless it is not clear, whether this process of horizontal gene transfer, apart from the transmission of oncogenes by viruses, has any biological consequences [252].

Garcia-Olmo et al. developed the “genometastasis hypothesis” in rats and concluded from their experiments that metastases are the result of a transformation of susceptible cells by CNAs [253,254]. According to their observations the hematogenous dissemination is closer associated with the detection of free-circulating tumor-derived DNA than with circulating tumor cells [255,256].

In summary, one can conclude that the observations mentioned above point to the possibility that circulating nucleic acids could have a biological meaning, but whether they are real information transmitters has to be determined in future experiments.

12. Can we expect to find the same alterations in CNAs as in tumor cells all the time?

Frequently there is no complete concordance between alterations found in the primary tumor and in CNAs and the reasons for these discordant results are manifold: the extracellular nucleic acids are a mix derived from normal and from tumor cells, most solid tumors are heterogeneous and contain subclones which are genetically different [84,99,257,258], and primary tumors, their subclones, and metastases may differ in the ability to shed nucleic acids into the environment (examples for discordant findings between tumor DNA and alterations in CNAs are listed in Table 7, see www.cnaps.net).

The quantity of tumor-derived DNA was determined in several papers and varied from less than 3% to more than 90% [202], and from 0.02% to 8%–11% [62]. Whether the

Table 7
Discordant alterations in tumor cell DNA and CNAs

Tumor entity	Marker	Discordance frequency	Reference
Small cell lung cancer	Microsatellite alterations/LOH	28%	Chen et al. 1996 [115]
Head and neck cancer	Microsatellite alterations/LOH	5%	Nawroz et al. 1996 [375]
Clear cell renal carcinoma	Microsatellite alterations/LOH	15%	Goessl et al. 1998 [374]
Breast cancer	Microsatellite alterations/LOH and p53 mutation	56%	Silva et al. 2002 [157]
Lung cancer	Microsatellite alterations/LOH	37%	Allan et al. 2001 [297]
Breast cancer	Microsatellite alterations/LOH	50% to 100%	Chen et al. 1999 [118]
Non-small cell lung cancer	Microsatellite alterations/LOH	22% to 39%	Sozzi et al. 1999 [117]
Melanoma	Microsatellite alterations/LOH	32%	Fujiwara et al. 1999 [371]
Bladder cancer	Microsatellite alterations/LOH	73%	Utting et al. 2002 [131]
Breast cancer	Microsatellite alterations/LOH	87%	Wang et al. 2003 [136]

application of different detection methods and/or target genes is the reason for the large variety or whether it is a reflection of the different biological behavior of different tumors is not known so far. When microsatellite alterations were analyzed, only 1/5 snap frozen tumor tissues tested positive, while all 5 samples tested positive after microdissection [259], demonstrating that the amount of DNA harboring these changes is of importance.

It is assumed that it takes 10 or more years for the development of a clinically detectable tumor and it is estimated that during this period several thousand [260] or even some million mutations are accumulated [261], leading to the development of genetically different tumor subclones. Garcia et al. were the first to explain the heterogenous pattern of MA in plasma compared to tumor DNA with the existence of different malignant clones, shedding their DNA into the circulation [262]. A genetic heterogeneity was described for tumors originating in the colon [257,263], prostate [264], and squamous cell carcinoma of the lung [265] and similar observations were made in lung and colorectal cancer for the presence of allelic imbalance (AI) represented by an LOH or microsatellite instability [56,266]. It is assumed that there are tumor cells left in the body of patients even after a complete therapy response and that these cells are able to survive for many years. These tumor cells keep on evolving new genetic traits, which can be detected in CNAs as demonstrated by Moriyama et al. in pancreatic carcinoma patients [259]. The analysis of hypermethylated sequences in lung cancer survivors, cancer-free smokers and a group of never-smokers showed little agreement when the results from sputum and plasma were compared [267], which is not surprising given the different anatomic compartments providing the DNA source and the contribution of other diseases to the DNA pool recovered in blood. Another explanation for the existence of discordant results could be the different access of the tumor subclones to the vascular system and/or a different ability to shed tumor DNA into the circulation [268]. The above mentioned considerations lead to the question whether a marker panel which is useful for the detection of alterations in tumor cells can also be applied for the examination of CNAs. This question in mind, Muller and coworkers developed a new approach to establish a marker set specifically designed for the analysis of CNAs [269].

Some of the tumor-associated genetic alterations are not only found in the tumor itself, but have been detected in histologically normal tissue of cancer patients, in people having

a certain risk for the development of a tumor, or even in cells obtained from obviously healthy subjects. This was demonstrated for the p53 tumor suppressor gene [270] whose alterations are found in progenitor lesions of the lung, esophagus, head and neck, and colon [271]. The presence of tumor-associated alterations has been described in histologically normal or minimally altered bronchial epithelium of chronic smokers [272], in sputum cells of patients with moderate asthma [483] and sarcoidosis patients [273], and in sputum cells of smokers who developed COPD [274]. A promoter methylation for p16INK4A and DAP kinase genes was frequently found in the bronchial epithelium from current and from former smokers, while no methylation was detected in bronchial epithelium from never-smokers [158] and similar findings were made for sputum DNA [275,276]. Patients with long-standing ulcerative colitis are frequently tested positive for mutations of the Ki-ras and p53 genes even if there are no signs of a tumor [277,278]. Some of these tumor-associated changes were also detected in plasma DNA of non-tumor patients. Khan et al. used a set of microsatellite markers to compare plasma DNA from lung cancer patients and patients with other respiratory diseases and found that 42% of the latter group tested positive [279], and Belinsky et al. detected gene promoter methylation in plasma from 10/74 women who never smoked [267].

In summary, taking into account the biological properties of a tumor, like the very long time for the development from a single transformed cell into a clinically detectable tumor, the presence of tumor subpopulations, the genetic heterogeneity of a tumor and its ongoing evolution, and the observation that some of the so-called tumor-associated alterations are in fact not tumor specific, but can be detected in a patient population without a malignancy, it is very unlikely that there will be detection assays, which are 100% sensitive and 100% specific at the same time. Additionally, it is not clear (i) which of the observed alterations in tumor cells are necessary for a malignant transformation, (ii) with what frequency genetic alterations occur in individuals who never go on to develop a disease, (iii) whether the detection of such alterations can be used to identify individuals with treatable neoplastic disease, (iv) whether individuals with pre-neoplasia and with a high(er) risk of subsequent disease may benefit from increased clinical observation or chemoprevention strategy, and (v) whether control patients who demonstrate genetic alterations in their

plasma DNA may represent a high-risk group for the development of a malignant disease.

13. Some technical and methodological aspects

There is no universally valid answer to the question as to whether plasma or serum is better suited for the analysis of cell free nucleic acids. The DNA yield from serum is higher than that from plasma, probably due to clotting and release of DNA from destroyed white blood cells [42,58,280,281]. This “contamination” with DNA from normal cells can have an influence on the results as was demonstrated by Hibi et al. who looked for genetic alterations in the sera of colorectal cancer patients, and found point mutations in the K-ras and p53 genes in some cases, but were unable to detect any LOH [282].

The number of protocols applied for the isolation of extracellular DNA and RNA are probably as numerous as the laboratories using them. Even if most researchers use commercially available kits (which have as many advantages as drawbacks), there is no accepted “standard” method so far [283]. The main problem when working with extracellular nucleic acids is the low quantity and their poor quality, irrespective of the isolation procedure applied. When a very crude extraction method was used, almost 50% of the PCR reactions failed, even if high DNA concentration (10 to 40 µg/mL plasma) were measured [284]. The use of kits makes the isolation step more reliable, but part of the DNA is lost, since the columns do not bind small DNA molecules (up to 150 bp) very efficiently [285,286].

Different preanalytical factors, like a delay in blood processing and storage temperature have an influence on the DNA yield [281]. When EDTA-stabilized blood samples were stored for 8 h at room temperature (RT) or for 24 h at 4 °C, there was no change in the extracellular DNA concentration. In contrast, the storage of blood samples without any additives led to a 3- to 5-fold increase of serum DNA upon storage at RT [287].

One of the most important critical factors is to free plasma/serum samples and all other body fluids from any “contaminating” cells [288–290], which can be achieved by centrifugation (combining low and high speed centrifugation) and/or filtration [289]. Lui and coworkers observed that a centrifugation of blood samples with a speed of up to 16.000×g did not result in a damage of blood cells and release of nucleic acids [291]. The choice of anticoagulant used for plasma preparation has no influence on the quantity of free-circulating DNA, only if there is a delay in the blood processing protocol, EDTA proved superior to citrate and heparin [292].

Whether storage of frozen samples (plasma/serum) has any effect on the DNA or RNA yield has not been analyzed extensively so far. Kopreski et al. were able to detect intact mRNA after 2 years of storage at –70 °C [186] and K-ras mutations in plasma DNA after more than 6 years [293]. Lee et al. collected serum and amniotic fluid samples and stored them at –20 °C for a different length of time [294] and determined that the amount of fetal DNA in the serum samples declined by a factor of approximately 0.66 genome equivalents/mL per month of storage, whereas in the amniotic fluids there was no DNA

loss found. Andriani et al. found yearly decay rates of approximately 30% for stored isolated DNA and DNA from stored plasma as well [295].

Sieben et al. showed a significant relation between the DNA input and the occurrence of LOH and microsatellite instability artefacts, and demonstrated also that the use of poor quality template DNA led to an increase in PCR artefacts [296]. The use of less than 10 ng DNA in a PCR may lead to allele dropout and false positive LOH results [118] and these results were confirmed by others [57,297]. Contrary to these observations, others found that the amount of 0.5 ng DNA/tube resulted in a false positive rate for LOH of 0.6% only [136]. Some authors prefer to increase the amount of template DNA for making sure that the PCR results are not an artefact [298,299], while others take the opposite direction and dilute the template up to a point of less than one molecule per tube [300]. Using multiple tubes for the same sample to obtain a reliable genotype [301] might solve the problems in some cases, but is not feasible for multi-target assays. According to a probability model, it is necessary to have at least 500 genome equivalents/PCR to be able to detect a tumor DNA provided this constitutes 1% of the total DNA [302]. Other factors that can influence the result of a PCR are the choice of DNA polymerase [303] and the template. The analysis of DNA from cell-free supernatants of urine, serum and plasma yielded 2- to 5-fold more alterations than the cell sediments [304]. A similar observation was made when DNA from plasma or cell-free bronchial lavage supernatants were compared to cellular DNA [299]. That the analysis of cell-free DNA might be of additional benefit was also shown by Swisher and colleagues, who detected more patients with ovarian cancer upon examining p53 mutations in plasma DNA (93%) compared to the cytological results [305]. and similar results were published by other groups [306,307]. In addition, more p53 and K-ras mutations were detectable in the cell-free pancreatic juice supernatant from cancer patients than in the cell sediment and the combination of the two analyses yielded a positivity rate of 100% while cytology was negative [308].

Most of the extracellular DNA has a low molecular weight. Li et al. isolated plasma DNA from pregnant women, size fractionated it by gel electrophoresis and used several microsatellite markers to characterize the high and low molecular weight DNA fractions. They demonstrated that the DNA fraction of a molecular size of <0.3 kb is mostly of fetal origin, while fragments >1 kb are mainly from maternal DNA [309]. Whether there is a comparable difference between “normal” and tumor-derived extracellular DNA in cancer patients is so far unknown.

The comparison of different labeling techniques (radioactivity vs. fluorescence) for the detection of microsatellite alterations in extracellular DNA seemed to have an advantage in one study [118], while others reported the same result with both methods [310].

In summary, it is fair to state that in order to make results comparable, we need to optimize and standardize methods for processing and storing of body fluid samples, for the isolation of nucleic acids and agreement on the best assays to detect and characterize tumor-associated alterations.

14. Conclusions and possible future developments

Due to impressive technical improvements in the past few years, large scale expression methods using microchips were developed and applied for the characterization of tumors [311]. In contrast, there has so far only one paper been published in which this technique was used for the expression analysis of free-circulating RNA [312]. In another study, RNA from cell-free saliva supernatant was used for profiling the salivary transcriptome aimed at the detection of oral cancer [313]. Whether such an analysis will lead to the identification of biomarkers specific enough to be used for cancer screening assay has to be determined [314].

The fact that it is possible to isolate extracellular nucleic acids from easily accessible plasma/serum and otherwise “useless” body fluids like lavage fluids (obtained from different organs), urine, stool, and ascites make them an extremely valuable source for future research projects. Also the observation that tumor-derived nucleic acids seem to be enriched in extracellular fluids and that these free-circulating nucleic acids are a reflexion of all physiological and pathological processes going on in the human body make them a very interesting target for the development of clinical useful assays. This has been demonstrated by the exciting finding that in some analyses a correlation between the detection of molecular alterations in CNAs and clinical data such as prognosis and response to therapy could be found. Most promising and potentially useful as a diagnostic marker is the observed close association between the detection of viral nucleic acids and tumor diseases especially in southern Asia. Provided that we will be able to find and explain the basic mechanisms behind CNAs, we will be able to exploit them for the development of biologically and clinically meaningful tools.

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