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Invited reviews

Circulating tumor DNA and its clinical applications

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Tumor-derived genetic changes and epigenetic alterations have been found in the serum and plasma of cancer patients. The alterations found in the blood mimic the alterations found in the primary tumor with a 71% of similarity. Moreover, these alterations are not found in normal tissues of healthy controls. These findings have important consequences for the detection, monitoring, prognosis, and early diagnosis of cancer. In this work, we will revisit anecdotal but repetitive observations of these possibilities that have been made during the last decade. New studies should be undertaken in order to ascertain the applicability of these observations, and their use in clinics. Sensitivity and specificity must be improved, and clinical and epidemiological studies should incorporate this technology to enable proper conclusions.

DNA nowotworu krążące w osoczu/surowicy i jego zastosowania kliniczne

W surowicy i osoczu chorych na nowotwory złośliwe można stwierdzić zmiany genetyczne i przemiany epigenetyczne, wywołane samą obecnością guza nowotworowego. Przemiany, stwierdzane we krwi, naśladują przemiany obecne w pierwotnym guzie, z ujednoliceniem sięgającym 71%. Co więcej, przemian tych nie stwierdza się w tkankach zdrowych osób badanych w ramach grup kontrolnych. Zjawiska te mają niebagatelne znaczenie dla wykrywania, monitorowania i określenia rokowania w poszczególnych przypadkach raka, jak również mogą być istotne przy wczesnym wykrywaniu nowotworów. W niniejszej pracy omówimy nierzadko anegdotyczne, niemniej jednak powtarzalne zjawiska, które zostały zaobserwowane w ciągu ostatniego dziesięciolecia. Należy podjąć badania mające na celu potwierdzenie przydatności opisanych zjawisk oraz zastosowanie ich w pracy klinicznej. W celu wyciągnięcia prawidłowych wniosków należy przede wszystkim poprawić czułość i specyficzność wspomnianych parametrów oraz uwzględnić je w badaniach klinicznych i epidemiologicznych.

Key words: serum or plasma tumor DNA
Słowa kluczowe: nowotworowe DNA krążące w osoczu/surowicy

Introduction

In 1869, Thomas R. Ashworth, at that time Resident Physician at Melbourne Hospital, reported the presence of tumor cells in the blood of a deceased cancer patient [1] observed under a simple microscope. Half a century ago, the hypothesis of the systemic character of cancer enabled the development of chemotherapy to complement local treatments, and the restriction of mutilating non-curative surgeries, as in the case of Halsted mastectomy in breast cancer [2]. Now, tumor cells can be detected in the blood of cancer patients at a concentration of one cell in 1 mL of blood, by a mixed technique of immunomagnetic enrichment, flow cytometry and immunocytochemical analysis [3]. In 1977, Leon et al. reported the presence of free DNA concentrations between 2 μg/mL in the serum of diverse neoplastic diseases, and they related the amount of circulating DNA with stage and response to treatment [4]. Small amounts of free DNA circulate in plasma or serum in both healthy individuals and in diseases other than cancer, but higher concentrations are present in cancer patients. Median concentrations of this DNA are reported to be between 24-211 ng/mL in tumor diseases [4, 5]. Tumor cells are characterized by specific genetic alterations. When such genetic alterations are identified in body fluids as serum or plasma, regardless of the presence of detectable tumor cells, it has been called free-circulating tumor associated DNA [6].

With the improvement of molecular analysis techniques, several studies have been conducted to detect the molecular events present in the serum that support evidence of tumor origin. The most frequent alterations studied are: microsatellite alterations, expressed as a loss of heterozygosity (an allelic loss) (LOH) or microsatellite instability (MI); the presence of mutations in specific oncogenes or in tumor suppressor genes; and the hypermethylations of the promoter regions of different genes. Microsatellite instability has been identified as one of the most important mechanisms of carcinogenesis and has

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first been described in colon cancer [7, 8]. Transcriptional silencing by methylation of CpG nucleotides containing the promoter regions of tumor suppressor genes seems a common characteristic of tumors and is being studied in various diseases. All of these molecular studies have been done in DNA obtained either in plasma or in serum. It is unclear which one better reflects the clinical status. Some studies have approached this problem. It seems that the concentration of cell-free genomic DNA in fresh plasma could be the same as the one found in circulation, and that serum must be collected with sample tubes without anticoagulant to allow clot formation [9]. For some authors, the levels of serum DNA or plasma DNA can be correlated with some different aspects of diagnosis or prognosis [10]. For the purpose of this review we will not differentiate between plasma and serum results, and we will refer to them indistinctly as s/p DNA.

The study of s/p DNA has opened up numerous areas of investigation and new possibilities for molecular diagnosis. We will divide this review into 5 parts: first, the etiology and origin of circulating free DNA; second, the similarities of alterations found in s/p DNA and primary tumor; third, its value as a prognosis tool to calculate the risk of local or distant recurrence and its relationship with survival; fourth, its value for follow-up patients or to evaluate response to therapy; and finally, the possibility to use it as an early diagnostic tool in an asymptomatic population.

Etiology

All individuals present circulating DNA in their blood, but the level of DNA in cancer patients is higher. Only a fraction of the circulating DNA found in neoplastic patients is attributed to cancer cells. The presence of tumor DNA using nucleic acid-base molecular analysis has been found in samples of ascites in ovarian cancer [11], stools in curable colorectal cancer [12], urine in bladder cancer [13], and sputum in lung cancer[14]. This has been attributed to a direct shedding of cells to stools in the case of colorectal primaries, or to sputum in the case of lung cancer etc.

The presence of nucleic acids in plasma and serum of neoplastic patients that harbor the same primary tumor-derived genetic or epigenetic alterations and viral nucleic acids has been related to the presence of tumor cells in the blood. These cells could be normal cells that have left the primary tumor but are still unable to metastasize to new organs, or they could be nonviable (apoptotic) neoplastic cells released as a consequence of anaplasia and necrosis of aggressive tumors. However, the possibility of the active shedding of DNA in the blood by malignant cells has also been suggested [15]. A recent study of 30 unselected cancer patients ruled out the possibility of lymphocyte DNA or degeneration of endothelial cells as origins of s/p DNA. The authors concluded that non-tumor DNA originates from normal cells that surround the tumor tissue, which are degenerated by the growing tumor, and that the soluble DNA in the form of chromatin fragments is released from apoptotic and necrotic cells. Therefore, the source of DNA would be the cells that disintegrate by apoptosis and/or necrosis in an expanding tumor tissue [16]. The origin of the apoptotic and necrotic cells would relate the presence of s/p DNA with the anaplasia and necrosis that is frequently associated with bad prognostic factors like high grade, non-differentiation, larger tumor and advanced stage. Moreover, it would explain the presence of DNA in tumors that infrequently give metastasis, as glioblastoma multiforme, but have a high degree of anaplasia and necrosis in spite of being confined to the brain [17]. DNA could simply be released from cell fragments of tumor tissue, which cross the blood-brain barrier by the rich brain vascularization and reach the blood stream but cannot metastasize to new organs. In Figure 1, a methylation-specific PCR of MGMT, p16, DAPK and RASSF1A done in serum and primary tumors of glioblastoma multiforme patients is shown.

However, origin from apoptotic and necrotic DNA would be difficult to explain in the case of earlier tumors – of small size with a low necrosis pattern, or in cases where tumor DNA is detected months before the diagnosis of cancer. Hence, the origin of circulating DNA remains unresolved.

Tumor origin of s/p DNA

To date, several studies have reported the presence of free DNA concentrations in the serum of cancer patients, and molecular studies have provided evidence that this DNA is similar to tumor DNA. A summary of different studies is detailed in Table I. In all these studies, the authors select patients with a particular cancer, individuals described as healthy controls, and a molecular alteration to be tested. Using PCR technology methods, the alterations found in tissue samples of various neoplastic diseases such as breast, head and neck, non-small- cell- and small-cell lung cancers, pancreas, liver, colon, kidney, esophagus, ovary, bladder, melanoma, and brain (glioblastoma) were definitely the same as the alterations found in s/p for the same patients [5, 11, 17-39]. Control healthy cases do not show any of the alterations found in cancer patients, confirming the specificity of circulating free DNA with neoplastic characteristics only in neoplastic patients. The range of similarity between the data found in tissue and serum/plasma is between 23-95%, with a median value of 71%, which means that depending on the specificity of a particular molecular alteration for every particular tumor, the serum or plasma is a good non-invasive method to study the alterations found in tumors. A major problem with these studies is the still low sensitivity of molecular analysis. The technique to obtain DNA in serum or plasma is important. Some authors have approached the issue of misinterpretations of results that occur when DNA concentrations are under the threshold of detection. PCR artifacts can mimic genetic alterations, especially with techniques like microsatellite analysis [40]. Therefore, s/p DNA must be

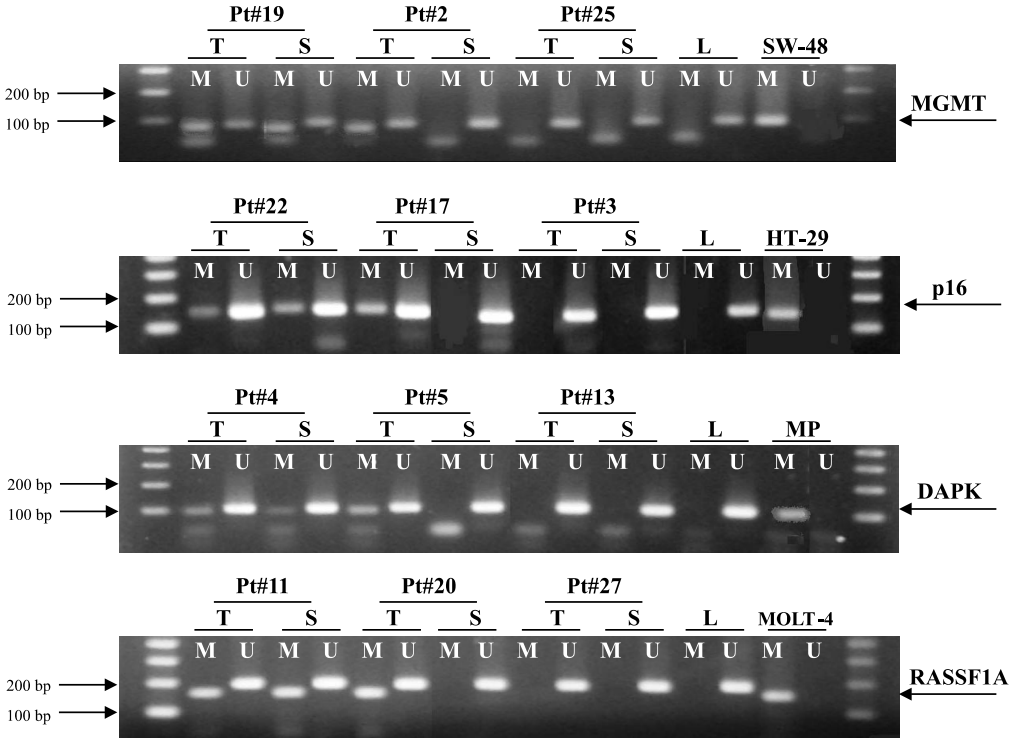

Figure 1. Methylation-specific PCR of MGMT, p16, DAPK and RASSF1A

Genomic DNA was obtained from tissue samples using DNA easy tissue kit (Qiagen, Hilden, Germany).
Serum DNA was extracted using the QIAmp Blood Mini Kit (Qiagen). DNA methylation patterns in the CpG dinucleotides of MGMT (GenBank accession number U95038), p16 (GenBank accession number X94154), DAPK (GenBank accession number NM_004938), and RASSF1A (GenBank accession number XM_004938) were determined by MSP assay.
Bisulfite-treated DNA was purified using the Wizard DNA clean-up system (Promega Co., Madison, WI, USA).
Five μl of bisulfite-modified DNA was amplified by PCR using primers that were specific for methylated or unmethylated sequences of each gene.
T= Tumor DNA; S= Serum DNA; L= Lymphocyte DNA used as negative and positive control for methylated and unmethylated PCR reaction, respectively; SW-48, HT-29, MOLT-4 cell lines and MP (*in vitro SssI* modified placental DNA) used as positive and negative control for methylated and unmethylated PCR reaction respectively.

analyzed with very sensitive and specific methods, such as mutant allele specific amplifications that exclude artifacts. When discrepancies exist between s/p DNA and tumor DNA (especially when a positive value in plasma is not found in tissue), good explanations could be: a characterization in tumor failed because of an excessive contamination of tumor tissue by normal cells, or because a sub clone of tumor cells with the alteration could release DNA in the plasma at higher concentrations compared to other clones in the tumor [40].

Once a tumor origin is confirmed, new possibilities in the monitoring of cancer can be foreseen. Several authors

Table I: DNA alterations found in tumor and serum or plasma in different tumors

Tumor	Alterations	Patients / controls	% Tumor positive	% S/P vs tumor	Ref.
Breast	LOH, P53, p16 methylation	62/17	90	73	5
	Microsatellite instability, LOH	52/9	55	55	18
	LOH	71/9	31.3	95	19
	P53	126	36.5	65.1	20
Head and neck	Methylation of p16, MGMT, GSTP1, DAP Kinase	95/25	55	42	21
Non-small cell lung	Microsatellite alterations, FHIT	87/14	56	61	22
	P16 methylation, MGMT, GSTP1, DAP Kinase	22/-	68	73	23
	APC methylation	99/50	96	47	24
	Microsatellite instability, LOH 5	17/31	59	70	25
Small cell lung	Microsatellite alterations	21/-	76	71	26
	Microsatellite instability	11/31	73	87	25
Pancreas	K-ras	39/60	72	32	27
	K-ras	21/4	71	60	28
Liver	P16 Methylation	22/38	73	81	29
	Mitochondrial D-loop mutations	19	68	80	30

Table I (cont'd): DNA alterations found in tumor and serum or plasma in different tumors

Tumor	Alterations	Patients / controls	% Tumor positive	% S/P vs tumor	Ref.
Colon	K-ras	14/-	50	86	31
	P16	52/44	38	70	32
Renal cell	LOH 3p	21/10	71	61	33
	Microsatellite alterations	53/20	–	87	34
Esophagus	P16 methylation	38/-	82	23	35
	ErbB-2 amplification	31/10	31	83	36
Ovary	LOH, microsatellite instability	20/8	56-63	73	11
Bladder	LOH	27/-	56	63	37
	Methylation of P14ARF	27/-	56	87	37
Melanoma	LOH (10 loci)	40	85	68	38
Gastric	Methylation of	54/30			39
	DAP-Kinase		70.3	68.4	
	E Cadherin		75.9	75.6	
	GSTP1		18.5	80	
	P15		68.5	81.1	
	P16		66.7	77.8	
Brain (GBM)	Methylation	21			17
	MGMT		28.6	83.3	
	P16		61.9	92.3	
	DAPK		38..1	100	
	RASS1A		52.4	91	

T= Tumors; S/P= serum or plasma, GBM= *glioblastoma multiforme*

have considered this new tool to predict prognosis, to monitor early recurrence as a tumor marker, or to be used as an early diagnostic tool.

Value of s/p as a Prognostic Tool

Tumor-specific genetic markers can be assessed primarily in tumor biopsies. However, in advanced patients with metastasis at diagnosis, surgery is not always performed so the availability of tumor tissue for genetic assessment is limited. The detection of tumor-specific genetic markers at distant sites from the tumor, such as in the blood, provides a unique and valuable tumor genetic marker assay for diagnosis and prognosis. The presence of serum DNA has been correlated with histological features of tumor aggressiveness, and some alterations seem to be a negative prognostic factor in some tumors [5, 6, 21, 24, 27, 28, 32, 34, 37, 38]. However, not all findings support this relationship [22]. The studies that approached this issue and have uncovered interesting relationships between prognosis and s/p plasma alterations are summarized in Table II. The sensitivity of the test for diagnosis and prognosis purposes could be further increased by obtaining a panel of positive markers capable of detecting molecular changes in plasma DNA for each tumor [41, 42]. The presence of s/p DNA in patients with colorectal cancer has been associated with poor prognosis regardless of the alteration detected [6].

The hypermethylations of the promoter region of p14ARF have been identified as a prognostic factor in bladder cancer [37]. This alteration, found in 56% of the tumor samples and in 87% of paired analyzed serums, was related to poor prognosis factors such as multicentricity, tumor size, bladder muscle invasion, and higher stage. In breast cancer, one study showed that the presence of circulating plasma DNA was a poor prognostic factor associated significantly with ≥3 positive nodes, ductal infiltrating carcinoma, high histological index, and high proliferative Ki-67 index [5]. In another work, the mean concentration of plasma DNA was found to be 211ng/mL substantially different from the levels found in healthy women (21ng/mL). Moreover, the presence of p53 mutations in plasma was found to confer a poor prognosis and was associated with larger tumors, higher stage, lymph node involvement, and negative receptor status ($p<0.05$). After a median follow-up of 29 months, multivariate analysis revealed that plasma DNA p53 mutations were an independent factor of early relapse and lower survival [20].

K-ras codon 12 mutations have been related to poor prognosis in pancreatic cancer [27, 28]. In malignant melanoma, a study of 10 microsatellite markers showed at least one LOH in 85% of the 76 tumors studied, and 68% of patients repeated this alteration in plasma. A significant relationship was found between the number of microsatellite markers with LOH in plasma and a more advanced clinical stage of the disease [38]. Only the LOH of loci D33S1293 was significantly correlated to disease progression.

In head and neck cancer, the hypermethylation of the promoter region of DAP kinase in serum was related to more affected lymph nodes and a more advanced stage of the disease. Moreover, a tendency to present distant metastasis in the case of hypermethylation was signaled by the authors [21]. In colon cancer, the presence of circulating tumor DNA has been identified as a poor

Table II. Studies that suggest s/p DNA has a value as a prognostic marker

Tumor	Num patients	Alteration	Prognosis	Ref.
Bladder	27	Methylation promoter p14ARF	Poor Multifocentric foci Larger tumors Relapse	37
Pancreas	21	K-ras codon 12	Poor Larger tumors Worse prognosis after surgery	28
	39	K-ras codon 12	Poor Stage Metastasis	27
Breast	62	DNA plasma levels (LOH or p53 or $p16^{INK4a}$)	Poor Nodes Proliferative index	5
	126	P53	Poor Size Stage Node status Estrogen receptor status	20
Melanoma	76	Number of LOH detected	Poor Stage	38
Head and Neck	95	Methylation DAP kinase promoter	Poor Lymph node Stage	21
Colon	52	Methylation $p16^{INK4a}$ promoter	Poor Stage	32
	58	pDNA present	Poor 2 year overall survival 2 year recurrence	6
Brain (GBM)	21	MGMT methylation	Good More responses to BCNU therapy Longer time to progression	17
Renal	53	Microsatellite alterations 20 markers	Poor Stage	34

prognostic factor, and specifically the hypermethylations of the promoter region of $p16^{INK4a}$ in serum seems to be related with an advanced stage[32]. In lung cancer, the quantitative hypermethylations measure on tissue of the adenomatous polyposis coli (APC) promoter 1A mapped at the chromosome 5p21 have been related as an independent factor with worse survival in 99 patients who exhibit this methylation in their tissues in 95% of the cases. Forty-two paired serum were also studied, but exhibited a methylated pattern in only 47% of the cases and the relationship on survival was not significant. Not one of the 50 controls exhibited hypermethylation. The authors attribute the lack of significance to the special choice of samples they have done, and they concluded that APC methylation in s/p analysis appears to be a promising non-invasive tool as a prognostic factor in primary lung cancer [24].

MGMT promoter methylation has been detected in the serum of glioblastoma patients. This gene encodes the enzyme O^6 methyl-guanine-methyl transferase, which removes the monofunctional O^6 adduct from the DNA produced by drugs commonly used in the treatment of glioblastoma multiforme. When response to chemotherapy was broken down by methylation status in serum only, objective response plus stable disease was seen in 8/8 (100%) patients with methylated bands, and in 7/17 (41.2%) with unmethylated bands (Fisher's exact test, $p=0.008$). Therefore, the detection of hypermethylation of MGMT promoter could be a good test to predict chemotherapy response [17]. Microsatellite alterations in s/p DNA have been related to a more advanced stage in renal cancer [34].

Tumor progression is a dynamic event and the genetic changes that concurrently occur are ongoing. The most significant advantage of assessing plasma alterations, compared with direct analysis of tumor biopsies, is the ability to monitor disease progression and genetic changes without assessing the primary tumor (i.e. to repeat the biopsy) at different stages of the disease and along its evolution. The identification of a panel of positive markers for each tumor should be the first step in designing prospective trials, in order to evaluate sensitivity and specificity for prognostic purposes.

Value as a Follow-up Tool

The analysis of s/p DNA could be a good tool for cancer screening. The disappearance of s/p DNA levels,

Table III. Studies that suggest s/p DNA has value as a follow-up tool

Tumor	Num patients	Alteration	Observations	Ref.
Non-small cell lung	84	Microsatellite instability LOH	Surgical tumor free patients drop DNA levels, recurrence produces a reappearance of high levels of circulating DNA	43
	22	3p Microsatellite instability or LOH	1 stage I patient with an abnormality was identified before developing distant metastasis	44
Small cell lung	35	MI, p53	In 15 patients it was possible to find correlations between tumor response and disappearance of abnormal plasma DNA or tumor progression	45
Esophagus	30	ErB-2 amplifications	Elevated s/p DNA values if patient treated but not in pre-treated	36
Breast	41	LOH or p53 or $p16^{INK4a}$	Persistence of molecular alterations after mastectomy in patients with vascular invasion, affected nodes and higher histological grade	46
Pancreas	21	K-ras	Treatment resulted in disappearance of K-ras mutation in 67% of patients. Three patients with persistent levels showed early recurrence or progressive disease	28

the persistence of its levels after treatment, or the re-appearance of the molecular alteration during follow-up, could become fine tools to document how effective the treatment is, and to follow up the patients without invasive and expensive techniques.

Some studies, which are summarized in Table III, have approached these objectives and interesting observations have been made.

Sozzi et al. analyzed the levels of tumor DNA using a DNA quantifications assay and molecular characterization of tumor plasma DNA through the analysis of microsatellite alterations [43]. In a previous study, the authors have documented that 61% of non-small cell lung cancer patients showed allele shift and LOH at FHIT and other genomic loci in tumor samples, and also in plasma irrespective of tumor size or stage, suggesting that circulating DNA was associated with early phases of lung cancer development [22]. They subsequently discovered that the levels of DNA dropped after surgery, in 1-6 months, to the levels of healthy controls and that all patients with recurrent disease showed an increase in levels of s/p DNA, suggesting a re-growing tumor. Our group detected a patient with a stage I lung cancer who presented persistent elevated levels of serum DNA with microsatellite alterations after surgery and developed systemic metastases on follow-up [44]. In addition, in small cell lung cancer it has been possible to correlate a good tumor response with the disappearance of abnormal plasma DNA and the reappearance at the moment of progression [45].

In esophageal cancer, erB-2 amplifications were found in untreated patients but not in treated ones [36]. Molecular alterations, such as LOH or p53 or $p16^{INK4a}$ previously found in breast cancer [5], were found to have follow-up implications. The persistence of these alterations after mastectomy was associated with poor prognostic characteristics, such as vascular invasion, affected lymph nodes, and higher histological grade, which are strong predictors of earlier recurrence and lower survival [46]. Furthermore, a disappearance of the plasma codon 12 K-ras mutation in pancreatic cancer in pre- and post-surgical samples of 6 out of 9 patients (67%) was observed, and 3 patients with persistently positive mutation were likely to show early recurrence or progressive disease [28]. Again, only prospective trials will confirm these earlier results.

Value of s/p DNA for Early Diagnosis

Although the usefulness of this methodology to detect micrometastatic disease in earlier phases of the disease remains to be proved [19], circulating s/p DNA is associated with the early phases of tumor development in many tumors, and it has been suggested that it could be useful as a tool for screening and for diagnosis [18, 19]. The goal would be to identify a useful test to be applied in an asymptomatic population and to be used as a non-invasive diagnostic procedure. Some studies suggest this application and are summarized in Table III. Some genetic alterations found in circulating DNA have been suggested as good candidates for a marker of early detection. For example, a selective mutation in codon 249 of the p53 gene has been identified as a hotspot in liver cancer associated with the exposure to aflatoxins. This tumor is a major cause of cancer death in Gambia. The Gambian patients examined (53 cancer patients, 13 cirrhotic patients, and 53 healthy controls) presented this mutation in their plasma at significantly different frequencies. The test was strongly associated with liver cancer in this population and could possibly be a good marker for early diagnosis, to identify patients at risk. Besides, none of the 16 French patients not exposed to aflatoxins presented this mutation. Of course, a follow-up control of positive non-cancer patients would be compelling [47].

Plasma DNA alterations were detectable in 43% of pathological stage I non-small-cell lung cancers, and in 45% of tumors up to 2 cm in diameter, which suggest that an early detection by a non-invasive screening procedure would be possible in a population at risk, such

Table IV. Studies that suggest s/p DNA has a value as an early diagnosis tool

Tumor	Num patients	Alteration	Observations	Ref.
Liver cancer (LC)	53 tumors 13 cirrhosis 56 controls	P53 Ser-249	It was found in 36% of LC patients, 15% cirrhosis, and 6% controls. Strong significant association with LC in Gambian patients	47
Lung non-small cell	87 tumors 14 controls	Microsatellite instability, LOH in FHIT	43% plasma abnormalities in 40 stage I patients	22
Lung cancer	60	LOH	41% of sensitivity for cancer. 2 patients were positive for LOH several months before diagnosis	48
Breast	61	LOH	Some patients with T1 or Tis (2) presented plasma abnormalities	18
Pancreas	21	K-ras	Plasma alterations were found 5 to 14 months before firm diagnosis in 4 cases	49
Ovary	20	Microsatellite alterations / LOH	3 stage Ia patients exhibit new alleles in their serum	11

Tis: tumor *in situ*

as heavy smokers [22]. LOH of diverse loci seem to have a 41% sensitivity to detect lung cancer, and were present in plasma several months before the cancer diagnosis [49]. LOH as an early event in carcinogenesis has been detected in cases of small breast tumors and in situ carcinomas [31]. Curiously, K-ras mutations were found in the plasma of 17 out of 21 patients with pancreatic cancer (81%) and none in 8 healthy controls. In 4 patients, the mutation was found 5 to 14 months before the diagnosis of cancer while the patient was diagnosed with chronic pancreatitis and biopsies were negative for tumor, suggesting a role in the earlier diagnosis of this disease. A later study with more cases determined that plasma K-ras analysis was a highly specific but low sensitivity approach for pancreatic cancer. The low sensitivity was attributed to the fact that plasma K-ras mutation was found in only 9 of the 28 patients (32%) that exhibited this mutation in tissue, constituting 72% of the group of 39 pancreatic cancers [49].

An alteration that is found in only 9% of tumor samples in affected patients, as is the case of mitochondrial DNA alterations at the D-loop region (mtDNA) in colorectal cancer, would be a poor early prognostic test, although it is possible to detect the same alteration in the serum in 14% of the affected individuals [50]. Larger studies are required to further assess the relevance of these observations and to understand their applicability to clinical practice.

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