TP53 and *KRAS2* Mutations in Plasma DNA of Healthy Subjects and Subsequent Cancer Occurrence: A Prospective Study

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Abstract

In cancer patients, plasma often contains mutant DNA released by cancer cells. We have assessed the significance of plasma DNA mutations for subsequent cancer development in healthy subjects in a large longitudinal prospective study. The European Prospective Investigation into Cancer and Nutrition study was analyzed with a nested case-control design. Cases were nonsmokers or ex-smokers for >10 years and newly diagnosed with lung, bladder, or upper aerodigestive tract cancers or leukemia accrued after a median follow-up of 6.3 years. Controls were matched 2:1 for follow-up, age, sex, area of recruitment, and smoking status. *KRAS2* mutations were detected by mutant-enriched PCR and sequencing (n = 1,098). *TP53* mutations were detected by denaturing high-performance liquid chromatography, temporal temperature gradient electrophoresis, and sequencing (n = 550). *KRAS2* or *TP53* mutations were detected in 13 of 1,098 (1.2%) and 20 of 550 (3.6%) subjects, respectively, 16 of whom developed cancer on average after 18.3 months of follow-up. Among 137 subjects who developed bladder cancer, 5 had *KRAS2* mutations [odds ratio (OR), 4.25; 95% confidence interval (95% CI), 1.27-14.15] and 7 had *TP53* mutations (OR, 1.81; 95% CI, 0.66-4.97). There was a nonsignificant trend for association between *TP53* mutations and bulky adducts in lymphocyte DNA (OR, 2.78; 95% CI, 0.64-12.17). This is the first report of *TP53* or *KRAS2* mutations in the plasma of healthy subjects in a prospective study, suggesting that *KRAS2* mutation is detectable ahead of bladder cancer diagnosis. *TP53* mutation may be associated with environmental exposures. These observations have implications for monitoring early steps of carcinogenesis. (Cancer Res 2006; 66(13): 6871-6)

Introduction

Circulating free DNA (CFDNA) in the plasma has emerged as a source of genetic material of tumoral origin in a variety of cancers. Somatic DNA alterations in CFDNA include point mutations, hypermethylation, microsatellite instability, and loss of heterozygosity (LOH; reviewed in ref. 1). For example, a special mutation at codon 249 in the tumor suppressor gene *TP53* has been identified in 36% of African patients with hepatocellular carcinoma (2), with a concordance of 88% between plasma and tumor (3). This mutation is considered as the result of sequence-specific DNA damage by metabolites of aflatoxin, a well-known hepatocarcinogen that contaminates many components of traditional diet in sub-Saharan Africa. In the same population, *TP53* mutations were also detected in the plasma of some subjects with liver cirrhosis (15%) or no liver disease (3%; ref. 2). However, the clinical utility of CFDNA for mutation screening remains to be ascertained.

Several clinical studies have reported that mutations detected in tumor tissues may also be detectable in plasma DNA (1). However, thus far, only one population-based, prospective study on detection

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of *TP53* mutations in plasma DNA has been reported (4). In that study, *TP53* mutations were detected 1 to 5 years before diagnosis in four of eight cases of hepatocellular carcinoma patients from Qidong (China), an area of high exposure to aflatoxin and high prevalence of hepatitis B virus chronic carriage (4). Mutations in *KRAS2* have also been detected ahead of clinical diagnosis in the plasma of subjects considered at high risk for colorectal cancer (subjects with genetic predisposition or with previous history of cancer) as well as in patients referred to a colonoscopy clinic for symptomatic evaluation (5). Similarly, Allan et al. reported the presence in CFDNA of LOH at up to four different loci in patients attending a bronchoscopy clinic and presenting a variety of symptoms suggestive of lung cancer (6).

In this study, we have analyzed *KRAS2* and *TP53* mutations in CFDNA in a longitudinal study, in relation with the occurrence of different types of cancers potentially caused by environmental exposures. The Genetic Susceptibility to Air Pollution and Environmental Tobacco Smoking (GENAIR) study focuses on cancers of the lung, bladder, and upper aerodigestive tract (UADT; including pharynx, larynx, and oral cavity) and leukemias in nonsmokers and ex-smokers who have quitted for >10 years. GENAIR was conducted to elucidate the relationship among air pollution, environmental tobacco smoking, and genetic susceptibility (7, 8). It was designed as a case-control study nested into European Prospective Investigation into Cancer and Nutrition (EPIC), a multicenter European study of >520,000 healthy volunteers of both genders, ages 35 to 74 years, and recruited in 23 centers from 10 countries between 1993 and 1998 (9). A total of 550 subjects for *TP53* mutations and 1,098 for *KRAS2* mutations were analyzed.

Materials and Methods

Study design and subjects. EPIC is a multimember European study, coordinated by the IARC (Lyon, France), including >520,000 healthy volunteers recruited in 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom) corresponding to 23 recruitment centers (10). The cohort includes subjects selected in the general population of a specific geographic area, town or province, of both genders and mostly in the age range 35 to 74 years at recruitment. Recruitment took place between 1993 and 1998. Detailed dietary and lifestyle histories collected mainly through self-administered questionnaires plus a 24-hour dietary recall through person-to person interview (in a 10% sample), anthropometric measurements, and a 30- to 40-mL blood sample are available. Blood samples were stored for 2 to 5 hours at 5°C to 10°C until they were transferred and processed in local laboratories in each but one of the recruitment centers (where specimens were shipped for processing to another center in the same country). The plasma, serum, RBC, and lymphocytes were stored at −196°C in 500 μL straws (7, 8, 10). All questionnaire information is available in a computerized format. Informed consent were obtained from all participants (11).

The follow-up was based on population cancer registries in seven of the participating countries: Denmark, Italy, the Netherlands, Norway, Spain, Sweden, and the United Kingdom. In France, Germany, and Greece, a combination of methods was used, including health insurance records, cancer and pathology registries, and active follow-up through study participants and their next-of-kin. Mortality data were also obtained from either the cancer registry or mortality registries at the regional or national level. Follow-up was virtually 100% complete. We used the *International Statistical Classification of Diseases, Injuries and Causes of Death, 10th Revision*.

GENAIR is a case-control study nested within the EPIC cohort, aiming at studying the relationship between some types of cancers and air pollution or environmental tobacco smoke (12). Cases are subjects with bladder, lung, or UADT cancers or leukemia, all newly diagnosed after recruitment. Only nonsmokers or ex-smokers who had given up smoking at least 10 years before recruitment have been included in GENAIR. We have identified 1,074 cases who met the protocol criteria. The Malmo center decided not to allow the use of their blood samples but participated in the rest of the project. After exclusion of the 231 Malmo cases, 843 cases were available, including 487 with blood samples (for further details, see the Peluso et al. study). Preliminary analyses suggested an association with bladder cancer. Thus, we analyzed all bladder cancers (n = 124); in addition, we enriched the series by extending follow-up (13 cases were identified in addition to the 124 described by Peluso et al.). Of the remaining cases of lung and UADT cancers and leukemia, we only analyzed a random sample of 254 cases matched 1:2 with controls for *KRAS* mutations. Because of the more demanding technical requirements for mutation detection, *TP53* analysis was limited to a subset of 236 cases (including bladder cases) matched 1:1 with controls (Table 1). Matching criteria were gender, age (±5 years), smoking status (never or former smoker), site of recruitment, and follow-up time. Mean follow-up was 89 months (minimum, 1.8; maximum, 123). GENAIR has been approved by the Ethical Committee of the IARC and by the local ethical committees of the 23 centers.

Mutation detection. CFDNA was extracted using affinity columns (Qiagen, Hilden, Germany) from 300 μL plasma and eluted in 200 μL buffer (7). Mutation analysis was done using 10 μL plasma DNA in a 25 μL PCR according to previously described techniques. For *TP53* mutation analysis, exons 5 to 9 were amplified by PCR in three segments and analyzed by denaturing high-performance liquid chromatography (DHPLC; ref. 13; for primers and conditions, see Supplementary Table) followed by direct sequencing of regions generating abnormal chromatograms. We have shown that DHPLC could reliably detect amounts of mutant DNA representing between 3% and 12% of wild-type DNA depending on mutation type and sequence context (13). Temporal temperature gradient electrophoresis (TTGE) was used as an additional strategy to enrich in mutant-specific sequences before sequencing (14). PCR products corresponding to DNA heteroduplexes were excised from the gel, reamplified with nested primers, and characterized by sequencing. Whenever possible, the presence of a mutation was confirmed by restriction digestion with enzymes cutting

Table 1. Distribution of cancers among subjects of the GENAIR study tested for *TP53* and/or *KRAS2* mutations

Pathology	Total samples	*TP53*			KRAS2		
		Total samples tested (%)	Mutants (% of total tested)	OR (95% CI)	Total samples tested (%)	Mutants (% of total tested)	OR (95% CI)
Bladder	137	126 (92)	7 (5.5)	1.81 (0.66-4.87)	131 (95.6)	5 (3.8)	4.25 (1.27-14.15)
Lung	115	36 (31)	2 (5.5)	1.83 (0.36-9.44)	77 (67)	0 (0)	—
UADT	82	30 (36.6)	0 (0)	—	57 (69.5)	1 (1.7)	2.07 (0.23-18.7)
Leukemia	166	44 (26.5)	1 (2.3)	0.70 (0.09-5.61)	120 (72.3)	0 (0)	—
None	1,086	314 (30)	10 (3.2)	1 (Reference)	713 (65.7)	7 (1)	1 (Reference)
Total	1,586	550 (34.7)	20 (3.6)		1,098 (69)	13 (1.2)	

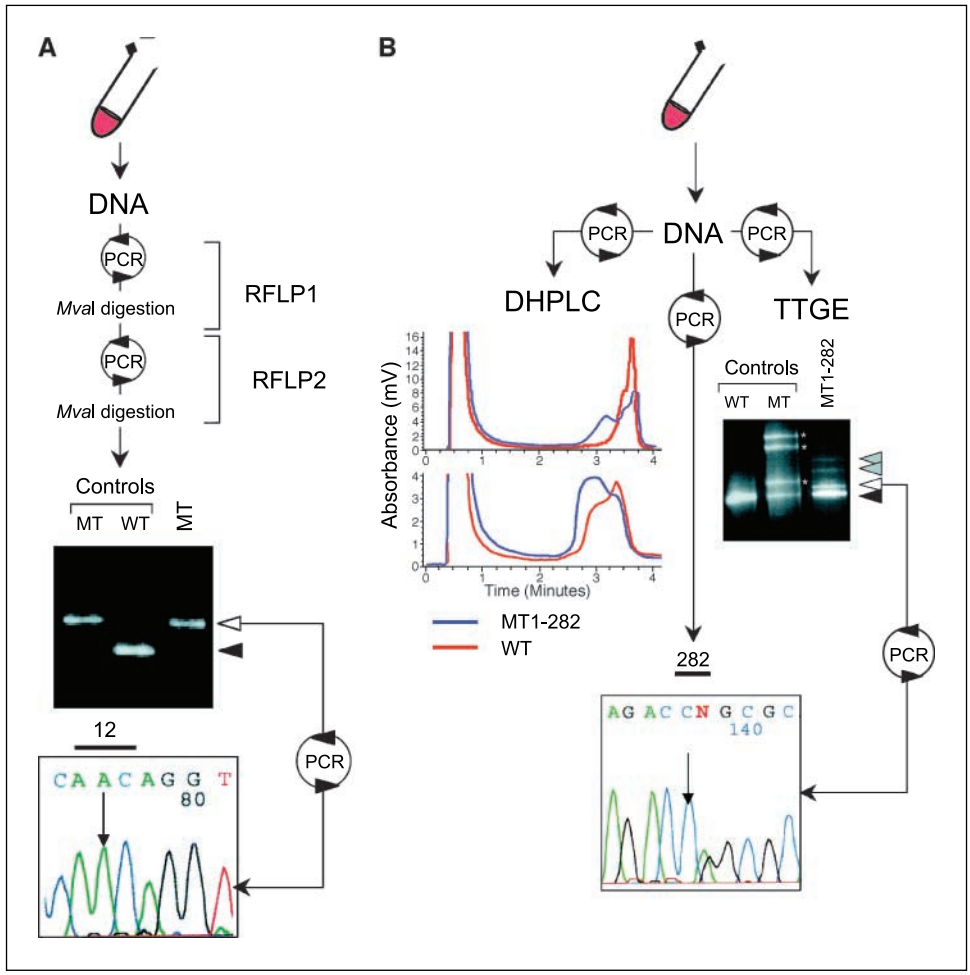

Figure 1. *KRAS2* (*A*) and *TP53* (*B*) mutation detection in CFDNA. *A,* detection of mutations in codon 12 of *KRAS2* by ME-PCR (involving two consecutive RFLP analyses for enrichment of the mutant DNA). After *Mva*I digestion, the mutant PCR product (*MT*; *white arrow*) is excised, amplified, and sequenced. *Black arrow,* wild-type PCR product (*WT*). *B,* detection of *TP53* mutation at codon 282. Mutations in exons 5 to 9 were first analyzed in CFDNA by DHPLC. Samples with abnormal DHPLC chromatograms were sequenced from an independent PCR product. If the mutation could not be characterized by sequencing, a new PCR product was analyzed by TTGE. If homoduplex products were detected they were excised from the TTGE gel, reamplified, and sequenced. All procedures were repeated using independent PCR products to rule out PCR artifacts. *Gray arrow,* mutant-wild-type heteroduplexes; *white arrow,* mutant homoduplexes; *black arrow,* wild-type homoduplexes; *white star,* mutant control heteroduplexes (*top two bands*) and homoduplexes.

within the suspected mutation site, allowing the detection of 3% to 6% mutant allele in a background of wild-type material (data not shown).

KRAS2 mutations at codon 12 were analyzed by mutant-enriched PCR (ME-PCR), involving two successive RFLP leading to enrichment in mutant sequence, and characterized by sequencing (ref. 15; see Supplementary Material for primers and conditions). To avoid false-positive results generated during successive PCR rounds, all analyses were repeated at least once. We have found that *KRAS2* codon 12 ME-PCR could detect up to 0.1% of mutant DNA in wild-type DNA. All mutations were confirmed by at least one second analysis and sequencing of independent PCR products. Results were scored as "positive" or "negative" with respect to established cutoff sensitivity values described above. However, no systematic attempt was made to obtain a quantitative assessment of the amount of gene mutation present in each sample.

Statistical analyses. We have computed odds ratios (OR) and 95% confidence intervals (95% CI) in logistic regression models. It was not possible to use conditional regression analysis for matched pairs because there was no pair in which both the case and the control(s) showed mutations. In addition to matching variables, we also fitted models, including educational level as a further adjustment variable. *P*s < 0.05 were considered as statistically significant. All analyses were done using SAS Statistical Package version 8 (SAS Institute, Cary, NC).

Results

Table 1 shows the distribution of cancer types and mutations among the subjects whose plasma was analyzed. A total of 550 specimens were tested for *TP53* (236 cases and 314 controls), whereas 1,098 specimens were tested for *KRAS2* (385 cases and 713 controls). The imperfect matching was due to the lack of biological samples for either member of the matched pairs.

The extraction and quantification of CFDNA has been reported elsewhere (7). The geometric mean of CFDNA concentrations was 28 ng/mL in controls and varied between 29 and 36 ng/mL in the various cancer groups, the difference being nonsignificant. Typical examples of *TP53* and *KRAS2* mutation detection are shown in Fig. 1. *KRAS2* mutations were tested in 1,098 subjects (Tables 1 and 2A and B). Six mutations were detected in cancer cases (median follow-up, 14.3 months; range, 2.6-24.9 months), five of which were bladder cancers (OR, 4.25; 95% CI, 1.27-14.15; Tables 1 and 2A). After adjustment for sex, age, area of recruitment, and education, an OR (95% CI) of 5.15 (1.34-19.72) for bladder cancer was found. Among subjects with mutations, there were six healthy controls (median follow-up, 85.5 months; range, 72.7-97.3 months) plus one control who developed skin cancer during follow-up (80.2 months). In the 550 subjects analyzed for *TP53* mutations (Table 1), 20 subjects had mutations at different codons, including 10 in cases, 7 of which were bladder cancers (Table 2A and B; OR, 1.81; 95% CI, 0.66-4.97). One bladder cancer case had two mutations at codons 207 and 216. With one exception (one subject with bladder cancer diagnosed 1.8 months after recruitment), mutations in CFDNA were detected at least 6 months ahead of diagnosis (median follow-up, 18.6 months; range, 1.8-44.8). For the 10 control subjects with *TP53* mutation, the median follow-up was 75.4 months (range,

Table 2.

Codon	Mutation classification*	Mutation	Amino acid change	Cancer site	Time to diagnosis (mo)
(A) Mutations detected in CFDNA of subjects who subsequently developed cancer					
KRAS2					
12		GGT>GTT	Gly>Val	Bladder	24.9
12		GGT>GTT	Gly>Val	Bladder	22.4
12		GGT>GTT	Gly>Val	Bladder	15.9
12		GGT>GTT	Gly>Val	Bladder	12.8
12		GGT>GAT	Gly>Asp	Bladder	2.6
12		GGT>GTT	Gly>Val	UADT	6.3
TP53					
175	MT1	CGC>TGC	Arg>Cys	Lung	19.1
179	MT2	CAT>CCT	His>Pro	Bladder	32.2
181	MT1	CGC>CAC	Arg>His	Lung	18.1
196	MT2	CGA>CAA	Arg>Gln	Leukemia	8.6
207 †	MT2	GAT>GGT	Asp>Gly	Bladder	44.8
216 †	MT3	GTG>GTA	Val>Val		
262	MT2	GGT>AGT	Gly>Ser	Bladder	18.1
271	MT2	GAG>GGG	Glu>Gly	Bladder	1.8
282	MT1	CGG>CAG	Arg>Glu	Bladder	15.8
324	MT2	GAT>GGT	Asp>Gly	Bladder	6.3
IN4 (13051)	MT3	G>C	None	Bladder	42.89
(B) Mutations detected in CFDNA of control subjects					
KRAS2					
12		GGT>GTT	Gly>Val		97.3
12		GGT>GTT	Gly>Val		85.5
12		GGT>GTT	Gly>Val		95.7
12		GGT>AGT	Gly>Ser		73
12		GGT>GTT	Gly>Val		80.2
12		GGT>GTT	Gly>Val		72.7
12		GGT>GTT	Gly>Val		89.4
TP53					
144	MT2	CAG>CGG	Glu>Arg		80.8
157	MT1	GTC>TTC	Val>Phe		64.3
163	MT1	TAC>TGC	Tyr>Cys		75.4
207	MT2	GAT>GGT	Asp>Gly		72.0
266	MT3	GGA>GGT	Gly>Gly		61.4
283	MT1	CGC>TGC	Arg>Cys		100.8
298	MT2	GAG>GGG	Glu>Gly		78
313	MT2	AGC>AAC	Ser>Asn		90.9
IN4 (13028)	MT3	T>C	None		82.6
IN6 (13436)	MT3	T>G	None		62.7

Abbreviation: UADT, Upper Aero-Digestive Tract.

*MT1, missense mutations frequent in IARC *TP53* mutation database; MT2, missense mutations infrequent in IARC database; MT3, mutations with no known effect on p53 protein (for details, see text).

†Mutations at codons 207 and 216 were found in the same plasma DNA sample.

61.4-100.8 months), and one of them developed pancreatic cancer after 82.6 months (mutation in intron 4, bp 13028). *TP53* mutations were classified in three groups. MT1 included missense mutations frequently reported in the IARC *TP53* database (ref. 16; at least 18 independent reports; http://www-p53.iarc.fr, version R10). MT2 included rare missense mutations. MT3 included mutations with no known effect on protein structure, such as mutations in introns (not at splice junctions) and silent mutations, none of which were registered as polymorphisms in the IARC *TP53* database. Taking into account only MT1 and MT2 groups, an OR (95% CI) for bladder cancer of 2.00 (0.66-6.06) was found, which was not significantly different than for any *TP53* mutation.

Recent publications have reported associations with biomarkers of environmental exposures in the GENAIR cohorts, including bulky DNA adducts in WBC (12), and biomarkers of genetic susceptibility, such as polymorphisms in genes involved in carcinogen metabolism, detoxification, and DNA repair (17).[32] Here, we have reanalyzed these previously reported data with respect to the presence of mutations in plasma DNA (Table 3). We found that *TP53* mutations, but not *KRAS2* mutations, were more

[32] H. Autrup et al. in preparation.

frequent among subjects with bulky adducts in WBC, although this association was not statistically significant (OR, 2.78; 95% CI, 0.64-12.17; Table 3). A tendency for association was found between the codon 399 *Gln* allele of *XRCC1* and *TP53* (A/A genotype; OR, 2.97; 95% CI, 0.96-9.14) but not *KRAS2* mutations. Mutations of *TP53* or *KRAS2* in CFDNA also showed a nonsignificant tendency for association with mutant alleles of *N*-acetyltransferase 2 (NAT2) defining the "slow acetylator" phenotype [*NAT2*5* and *NAT2*6*; OR (95% CI), 1.68 (0.63-4.49) for *TP53* and 2.66 (0.73-9.74) for *KRAS2*].

Discussion

Our results from a large prospective study, GENAIR, show that mutations in *TP53* or *KRAS2* could be detected in CFDNA of healthy subjects on average 20.8 months (range, 1.8-44.8) and 14.3 months (range, 2.6-24.9) before cancer diagnosis, respectively. The presence of a mutation was not dependent on the total amount and concentration of DNA extracted from plasma (geometric mean in samples with a *TP53* or *KRAS2* mutation, 28 ng/mL). The GENAIR study includes specimens collected in 10 different countries; in a previous study, we reported a variation in the amount of plasma DNA between participating centers. However, the distribution of mutations is too sparse to allow a comparison by center. The fact that each mutation in *TP53* or in *KRAS2* was confirmed by at least two analyses of independent PCR products ruled out that their detection was a polymerase fidelity artifact (18). We found that mutation in CFDNA had a tendency to predict bladder cancer but not other cancers (OR, 2.08; 95% CI, 0.90-4.76). However, the association with bladder cancer was significant only for mutations at codon 12 in *KRAS2* (adjusted OR, 5.15; 95% CI, 1.34-19.72) and not for *TP53* (OR, 1.81; 95% CI, 0.66-4.97), even when only mutations leading to amino acid substitutions were taken into account (OR, 2.00; 95% CI, 0.66-6.06). Furthermore, when comparing subjects with and without mutations, the follow-up times were significantly shorter for those subjects with mutation in *KRAS2* ($P = 0.006$) but not in *TP53* ($P = 0.35$; data given in Supplementary Material). This observation supports an association between plasma *KRAS2* mutation and the occurrence of bladder cancer.

Table 3. Association between mutations in CFDNA and DNA adducts or polymorphisms in susceptibility genes

	Mutant	Total	OR (95% CI)*
TP53 and DNA adducts			
Detectable	16	409	2.78 (0.64-12.17)
Nondetectable	2	122	1 (Reference)
KRAS2 and DNA adducts			
Detectable	0	779	0.49 (0.15-1.61)
Nondetectable	4	170	1 (Reference)
TP53 and *XRCC1*			
AA	4	69	2.97 (0.96-9.14)
AG	7	233	1.07 (0.37-3.13)
GG	7	234	1 (Reference)
KRAS2 and *XRCC1*			
AA	2	116	0.7 (0.12-4.07)
AG	5	419	0.82 (0.21-3.17)
GG	5	424	1 (Reference)
TP53 and *NAT2*			
Slow acetylator	12	525	1.68 (0.63-4.49)
Fast acetylator	5	410	1 (Reference)
KRAS2 and *NAT2*			
Slow acetylator	9	534	2.66 (0.73-9.74)
Fast acetylator	3	413	1 (Reference)

*Adjusted for age, sex, time and site of recruitment, and smoking history.

In bladder cancer tissues, *TP53* mutations are rather rare in early lesions but are common in advanced, metastatic cancers, with a prevalence of 50% in progressive, muscle-invasive disease (19). Mutations at codon 12 of *KRAS2* are infrequent in bladder cancer (20, 21). In a recent study, Jebar et al. have found *KRAS2* mutations in 3 of 98 bladder cancer patients. However, these mutations were early events, mutually exclusive to *FGFR3* mutations, suggesting their association with a subgroup of bladder cancers (22). Interestingly, experimental studies in transgenic mice have shown that tissue-specific expression of a *RAS* transgene in the urothelium led to urothelial hyperplasia and superficial papillary tumors (23). These observations suggest that activation of *RAS* may contribute to early steps of carcinogenesis in the bladder. Thus, the temporal sequence of occurrence of *KRAS2* (early event) and *TP53* (late event) during bladder tumorigenesis may explain our observation that *KRAS2* mutation in CFDNA is a better predictor of bladder cancer than *TP53* mutation. However, as tumor tissues were not available in the present study, we cannot ascertain whether the tumors also contained the same mutations as those identified in the plasma and whether these mutations occurred as early or late events.

TP53 and *KRAS2* mutations were detected in 3% and 1%, respectively, of subjects who did not develop cancer during follow-up (10 *TP53* mutations and 7 *KRAS2* mutations). The proportion of mutations in controls remains low compared with subjects who developed bladder cancer (5.5% for *TP53* and 3.8% for *KRAS2*). It should be kept in mind that controls were matched with cases for the duration of follow-up. Thus, it is possible that control subjects with mutations in CFDNA will ultimately develop a cancer disease after the period of matched follow-up. Of 65 controls subjects who developed cancer after follow-up, 2 had a *TP53* or a *KRAS2* mutation and were diagnosed with pancreas and skin cancer, respectively. These numbers are too small to be informative on the risk of cancer in positive, control subjects.

Another important information of the present study is that no predictive value of CFDNA mutations were found for several cancers other than bladder, including lung, UADT, and leukemia. This observation is in line with many studies showing considerable variations in the concordance between mutations detected in CFDNA and in tumors in cancer patients (1). Although some of these variations can be explained by technical considerations (e.g., the need for high sensitivity methods to detect mutations in CFDNA), they are most likely to reflect intrinsic differences among tissues in the way mutations occur, persist, expand clonally, and are released in CFDNA. In a recent study, we found an overall concordance of 88% between *TP53* mutations in CFDNA and in matched primary liver cancer in a series of patients from western Africa (3). In contrast, in lung cancers, recent studies have reported an overall poor concordance between mutations in CFDNA and tumor (24). Thus, the occurrence of mutations in CFDNA may have organ- and tissue-specific patterns that reflect the architecture of the organ (e.g., the proportion of released DNA that ends up in the bloodstream), the type and level of exposure to mutagens, and the

temporal sequence of occurrence of mutations in the target tissue. Thus, in bladder cancer, molecular alterations, such as mutations in *FGFR3* and microsatellite instabilities, which occur in bladder transitional cell carcinoma at higher frequencies than either *TP53* or *KRAS2* mutations (30-40% and up to 70% alterations respectively; refs. 25–27), may provide additional, sensitive markers for CFDNA screening.

In the GENAIR study, several genetic polymorphisms have been assessed in relation to cancer risk. The rationale for analyzing these polymorphisms as candidates is explained by Matullo et al. (17). Our results suggest a tendency for plasma DNA mutation to correlate with exposure and/or susceptibility to environmental mutagens. Two polymorphisms showed a suggestive tendency for association with CFDNA mutations. The codon 399 *Gln* allele (A/A genotype) of *XRCC1* is a potential biomarker of susceptibility to chemically induced genetic damage (28). The "slow acetylator" phenotype of NAT2, a carcinogen-metabolizing enzyme involved in the inactivation of arylamines, is suspected to determine an increasing risk for bladder cancer (29). Thus, mutations in the CFDNA of healthy subjects may reflect the effect of ongoing exposures to environmental carcinogens, particularly in subjects who have genetic polymorphisms that predispose to higher levels of mutagenic damage by such carcinogens. This interpretation is consistent with the results of studies on *TP53* mutations at codon 249 in the plasma DNA of healthy subjects from regions of high incidence of hepatocellular carcinoma in The Gambia, West Africa (3) and in Qidong, China (4). This mutation is considered as a "fingerprint" of mutagenesis by aflatoxins, and its presence in the plasma of subjects without cancer may reflect ongoing, dietary exposure to this carcinogen. In the present study, the potential exposures are more diverse and less characterized and pervasive as aflatoxin in The Gambia and in Qidong. Overall, these observations have implication for the use of CFDNA mutations as indicators of disease in prospective studies. The rarity of mutations in both cases and controls is a limitation for their use in clinical cancer detection. Further investigations are needed to determine whether it is possible to discriminate disease-associated from exposure-associated CFDNA mutations based on mutation patterns and quantitative accrual over time of mutant DNA in the plasma.

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