

# Genetic Analysis of DNA Excreted in Urine: A New Approach for Detecting Specific Genomic DNA Sequences from Cells Dying in an Organism

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**Background:** Cell-free DNA from dying cells recently has been discovered in human blood plasma. In experiments performed on animals and humans, we examined whether this cell-free DNA can cross the kidney barrier and be used as a diagnostic tool.

**Methods:** Mice received subcutaneous injections of either human Raji cells or purified <sup>32</sup>P-labeled DNA. DNA was isolated from urine and analyzed by measurement of radioactivity, agarose gel electrophoresis, and PCR. In humans, the permeability of the kidney barrier to polymeric DNA was assessed by detection in urine of sequences that were different from an organism bulk nuclear DNA.

**Results:** In the experiments on laboratory animals, we found that ~0.06% of injected DNA was excreted into urine within 3 days in a polymeric form and that human-specific *Alu* sequences that passed through the kidneys could be amplified by PCR. In humans, male-specific sequences could be detected in the urine of females who had been transfused with male blood as well as in DNA isolated from urine of women pregnant with male fetuses. *K-ras* mutations were detected in the urine of patients with colon adenocarcinomas and pancreatic carcinomas.

**Conclusions:** The data suggest that the kidney barrier in rodents and humans is permeable to DNA molecules large enough to be analyzed by standard genetic methodologies.

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Apoptosis (programmed cell death) is now among the most intensively studied phenomena in the biological and medical sciences. As a result of cell suicide, cellular components are dismantled and usually phagocytosed by macrophages or neighboring cells. During the early stages of apoptosis, nuclear DNA is degraded, producing nucleosomes and their oligomers. Taking into account that in human adults ~10<sup>11</sup> cells die daily, the amount of released DNA should be ~0.6 g. Some chromatin degradation products are further degraded into acid-soluble products and are reutilized within an organism, whereas others escape phagocytosis and appear in the bloodstream.

The presence of DNA in plasma has been described in many reports (1–4). Plasma DNA content appears to reflect the amount of cell death occurring in the whole body and is increased during destructive pathological processes, including cancer (1–3). However, interpretation of these early results is ambiguous. Because the amount of cell-free DNA in blood is very low, one can suggest that this DNA is a result of white blood cell destruction during serum or plasma preparation. Conclusive data that demonstrated non-blood cell origins of plasma DNA were obtained in 1996 by Chen et al. (5) and Nawroz et al. (6), who detected in the serum of cancer patients microsatellite DNA sequence alterations identical to those found in DNA isolated from tumors (lung, head and neck cancer) of the respective patients. Similar findings were obtained by other groups that used different tumor markers, including *K-ras* (7–9) or *p53* (10) mutations, Epstein-Barr virus DNA (11), and methylation abnormalities (12, 13). The appearance in plasma of DNA from cells dying in a body was also shown by detection of fetus-specific sequences in the plasma of pregnant women (14–17).

These results indicate that a portion of DNA from

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dying cells escapes intracellular degradation and phagocytosis, and circulates in the bloodstream in a polymeric form. This circulating plasma DNA is detectable by PCR and, therefore, can be used for genetic analysis [see Refs. (18–20)].

When analyzing the fate of labeled DNA injected into the mouse abdominal cavity, we found that a portion of this DNA was excreted into urine in an acid-insoluble form. The goal of our study was to determine whether cell-free DNA from the bloodstream crosses the kidney barrier in a form suitable for PCR analysis. Compared with tests for plasma DNA, urinary DNA analysis would be noninvasive, not restricted by sample volume, and readily performed.

In this report, we present data demonstrating that polymeric DNA fragments from dying cells can cross the kidney barrier and be used for genetic analysis.

### Materials and Methods

#### EXPERIMENTS WITH LABORATORY ANIMALS

Male albino C3HA mice received subcutaneous injections of either purified  $^{32}\text{P}$ -labeled  $\lambda$ -phage DNA (0.1–0.5  $\mu\text{g}$ /animal) or human Raji lymphoma cells ( $10^8$  cells/animal). Before the injection, Raji cells were irradiated with 1000 rad of  $^{137}\text{Cs}$   $\gamma$ -rays to induce cell death.  $\lambda$ -Phage DNA was labeled by nick translation (21) with [ $\alpha$ - $^{32}\text{P}$ ]dCTP to a specific radioactivity of  $\sim 10^8$  cpm/ $\mu\text{g}$ . The radioactivity of excreted DNA products was measured by scintillation counting. DNA from urine was purified by phenol treatment and ethanol precipitation, and DNA fragment size was determined by electrophoresis in a 1% agarose gel followed by autoradiography. Detection of human-specific sequences in mouse urinary DNA was performed as described previously (22).

#### PATIENTS

Urine samples from pregnant women, at gestational ages of 7–10 weeks when deliberate abortions were undertaken, were obtained before abortion with informed consent from women attending the Moscow city antenatal clinic no. 7. Fetal sex was determined by PCR of DNA obtained from aborted tissues.

Samples of colon cancer and surrounding “normal” tissues were obtained during surgery of patients in the Cancer Research Center. Urine samples were obtained 24 h before surgery.

Because human urine contains a nuclease activity [Ref. (23), and our unpublished observations], samples (25–50 mL) were collected fresh (i.e., accumulated during morning hours). The first void of the day was never used. The samples were adjusted to 10 mmol/L EDTA and stored frozen before use. To control potential contamination of solutions and final probes with exogenous DNA, the experimental setup also included a control (25 mL of saline solution) that was carried through all subsequent procedures.

DNA was purified from nonfractionated urine samples

(i.e., not subjected to centrifugation) to avoid possible DNA losses via adsorption to particulate material. Urine samples (3–5 mL) were mixed 1:1.5 (by volume) with 6 mol/L guanidine isothiocyanate, and DNA was adsorbed on a Wizard column (Minipreps DNA purification system; Promega), as recommended by the manufacturer. Columns were washed with 500 mL/L isopropanol, and DNA was eluted with 200  $\mu\text{L}$  of distilled water.

#### Y-CHROMOSOME-SPECIFIC SEQUENCES

Nested PCR was performed using the following primers to amplify the Y-chromosome-specific, 3000–5000 times reiterated DYZ1 (24) sequence: (a) 5'-TCCACTTTATTC-CAGGCCTGTCC-3' (Y1) and 5'-TTGAATGGAATGG-GAACGAATGG-3' (Y2) to amplify a 154-bp sequence; and (b) 5'-GTCCATTACTACTACATTCCC-3' (nY1) and 5'-AATGCAAGCGAAAGGAAAGG-3' (nY2) to amplify a nested 77-bp sequence.

Target sequences were amplified in a 50- $\mu\text{L}$  reaction volume containing 5–10  $\mu\text{L}$  of DNA solution, 100  $\mu\text{mol/L}$  each dNTP, 0.5  $\mu\text{mol/L}$  each oligonucleotide primer, and 2 U of Taq polymerase. For nested PCR, 5  $\mu\text{L}$  of the first reaction mixture was introduced into a 50- $\mu\text{L}$  reaction volume. Thirty-five and 25 reaction cycles were performed for the first and second stages, respectively. Cycle conditions were as follows: denaturation at 94  $^{\circ}\text{C}$  for 60 s; annealing at 60  $^{\circ}\text{C}$  and 55  $^{\circ}\text{C}$  for 60 s for the first and second reactions, respectively; chain elongation at 72  $^{\circ}\text{C}$  for 60 s. The denaturation step was extended to 3 min at the beginning of the first cycle, and the chain elongation step was extended to 7 min in the last cycle.

To avoid contamination of reagents, the PCR mixture was treated, before DNA addition, with *Hae*III and *Eco*RI restriction endonucleases (5 U/25  $\mu\text{L}$  of reaction mixture, 3 h at 37  $^{\circ}\text{C}$ ) specific for the target sequence. The samples were then heated at 94  $^{\circ}\text{C}$  for 3 min in a thermocycler to inactivate the endonucleases, and DNA samples were added. PCR products were analyzed by electrophoresis in a 10% polyacrylamide gel and stained with ethidium bromide (0.5 mg/L).

#### K-ras MUTATIONS

K-ras mutations were detected by a two-stage PCR assay using selective restriction enzyme digestions of an artificially created site to enrich for mutant K-ras DNA (8). PCR was performed with oligonucleotide primers K-ras-L (5'-actgaatataaactgtggtagtggacct-3') and K-ras-R (5'-tcaaagaatggtctctggacc-3'). The first primer, which is immediately upstream of codon 12, is modified at nucleotide 28 (G to C) to create an artificial restriction enzyme site (*Bst*NI). The oligonucleotide K-ras-R is also modified at base 17 (C to G) to create an artificial *Bst*NI site to serve as an internal control for completion of the digestion. As a result, the nonrestricted PCR product was 157 bp long, whereas the product being restricted at both sites (wild-type sequence) was 113 bp long, and the product restricted only at the right site (the left site is modified by

mutation) was 142 bp long. The reaction mixture was cycled 15 times at 94 °C for 48 s, 56 °C for 90 s, and 72 °C for 155 s. A 10- $\mu$ L aliquot adjusted with 1 $\times$  *Bst*NI reaction buffer was digested with 10 U of *Bst*NI at 60 °C for 90 min. A 10- $\mu$ L aliquot of the digested PCR mixture was removed to a new tube, and a new reaction mixture was set up for the second amplification step (35 cycles of 94 °C for 48 s, 56 °C for 90 s, and 72 °C for 48 s) using identical constituents. A second *Bst*NI restriction digestion was performed using 25  $\mu$ L of the second-step PCR product at 60 °C for 90 min. The final digestion product was separated by electrophoresis through a 3% Nu-Sieve agarose gel or a 12% polyacrylamide gel.

#### MEASUREMENT OF DNA CONCENTRATION

Fresh urine samples were centrifuged 10 min at 800g, and DNA was isolated from the supernatant as described above. DNA samples were stained with 0.1 mg/L Hoechst 33258, and the DNA concentration was determined by spectrofluorimetry as described (25).

### Results

#### DNA CROSSES THE KIDNEY BARRIER IN RODENTS

Mice received injections of [<sup>32</sup>P]-labeled  $\lambda$ -phage DNA, and the appearance of radiolabeled DNA degradation products in the urine was quantified. The data presented in Table 1 show that most DNA degradation products are apparently reutilized and that only ~3.2% of injected DNA is excreted within a 3-day interval. An even smaller portion of injected DNA (0.06%) appeared to be acid-insoluble, i.e., relatively polymeric ( $\geq$ 15–20 nucleotides long). Agarose gel electrophoresis of the DNA fragments present in the urine showed a wide range of sizes with the predominant fraction being ~150 bp (Fig. 1).

These results suggest that most of the injected DNA is reutilized in an organism or degrades into acid-soluble products and is excreted in the urine. However, a small portion of this DNA is not completely degraded and is excreted from the bloodstream through the kidney barrier into the urine. It is this latter fraction that is the focus of our further investigation.

Of course, the excretion of naked DNA and DNA from

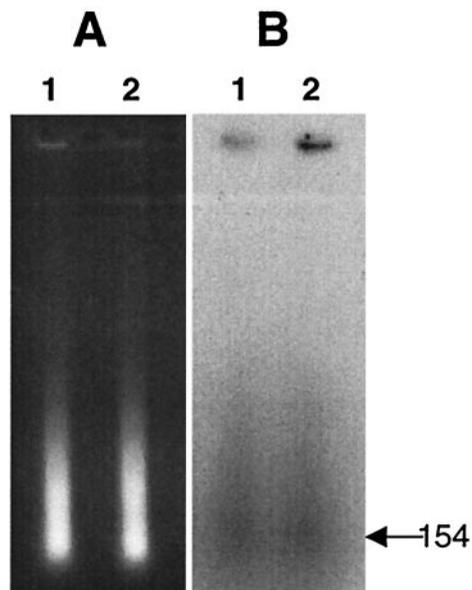


Fig. 1. Agarose gel electrophoresis of DNA isolated from mouse urine.

Mice received injections of nick-translated <sup>32</sup>P-labeled  $\lambda$ -phage DNA (10<sup>7</sup> cpm/animal). Urine from each mouse (n = 3) was collected for 3 days. DNA was isolated and purified by phenol treatment and then subjected to electrophoresis on a 1% agarose gel. Lanes 1 and 2, results of two independent experiments. (A), ethidium bromide staining to visualize DNA; (B), autoradiography to visualize excreted radiolabeled DNA.

dying cells can be different. The DNA from dying cells is apparently bound to multiple proteins and may be protected from nucleases better than the naked DNA used in the model experiments. At the same time, these DNA-bound proteins can affect, positively or negatively, the DNA transition through the kidney barrier. The following experiments carried out on mice were designed to see whether the DNA from dying cells is excreted in the urine and can be used as a template for PCR. Human Raji lymphoma cells were irradiated with 1000 rad of  $\gamma$ -rays to induce their reproductive death and were injected into mice. The urine was collected for 3 days; DNA was isolated and tested by PCR for the presence of human-specific sequences, using *Alu* oligonucleotide-directed PCR, which allows simultaneous screening of multiple loci (22). Fig. 2 shows that the band distribution pattern characteristic of human cells is also seen with DNA obtained from the urine of mice injected with the human cells but not with DNA from the urine of control mice that lack those sequences. Hence, DNA from dying cells is excreted in the urine, preserves its template function, and can be used for PCR analysis.

#### DETECTION OF DNA FROM DYING CELLS IN HUMAN URINE

An important question raised by this study was whether the results obtained with laboratory animals held true for humans. That is, does DNA that circulates in the human bloodstream penetrate the kidney barrier? To our knowledge, information on this subject is lacking. We found that DNA can be easily isolated from the urine of healthy

**Table 1. Excretion of injected [<sup>32</sup>P]DNA into urine.<sup>a</sup>**

Radioactivity	Amount of DNA excreted into urine			
	1st day	2nd day	3rd day	Total
Total				
cpm	1 080 800	100 800	7700	1 189 300
% of injected DNA	2.9	0.3	0.02	3.2
Acid-insoluble				
cpm	21 000	ND <sup>b</sup>	ND	21 000
% of injected DNA	0.06			0.06

<sup>a</sup> Each mouse received an injection containing 37  $\times$  10<sup>6</sup> cpm of nick-translated  $\lambda$ -phage <sup>32</sup>P-labeled DNA. Urine from each animal (n = 3) was collected daily to measure total and acid-insoluble radioactivity.

<sup>b</sup> ND, not detected.

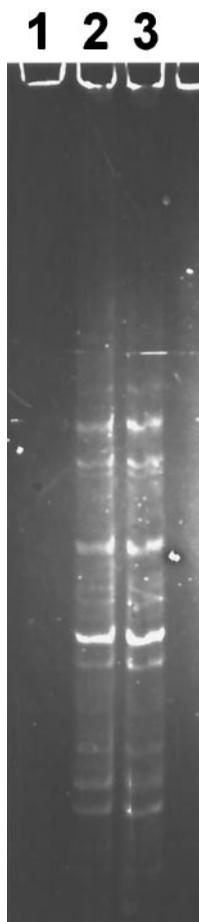


Fig. 2. Detection of human cell sequences in DNA from mouse urine. Human Raji lymphoma cells ( $10^8$ ) were irradiated with 1000 rad of  $^{137}\text{Cs}$   $\gamma$ -rays and inoculated subcutaneously into a mouse. Urine was collected over 3 days, and DNA was isolated and tested for the presence of human-specific sequences using *Alu* oligonucleotide-directed PCR (22). Lane 1, DNA from urine of a control mouse; lane 2, control Raji cell DNA; lane 3, DNA from the urine of a mouse inoculated with Raji cells.

individuals. The DNA concentration was 2–96  $\mu\text{g}/\text{L}$ . The DNA was similar in size to that in the rodent urine (Fig. 3).

Because many cells die daily in the kidney and bladder, to demonstrate that DNA also can cross the kidney barrier in humans, we selected three models that could discriminate two types of DNA in urine: excreted DNA (i.e., originating from cells dying in various tissues of an organism), and locally degraded DNA (i.e., originating from cells dying in the urinary tract itself). We first tried to detect Y-chromosome-specific sequences in the urine of women who had been transfused with blood from male donors. We then tried to detect Y-chromosome-specific sequences in the urine of women bearing male fetuses. In these two models, detection of male-specific sequences in the DNA isolated from urine would unequivocally indicate that polymeric DNA can penetrate the kidney barrier (as well as the placental barrier in the second model). Finally, we analyzed the presence of *K-ras* mutant sequences in the urine of patients with colorectal and pancreatic carcinomas.

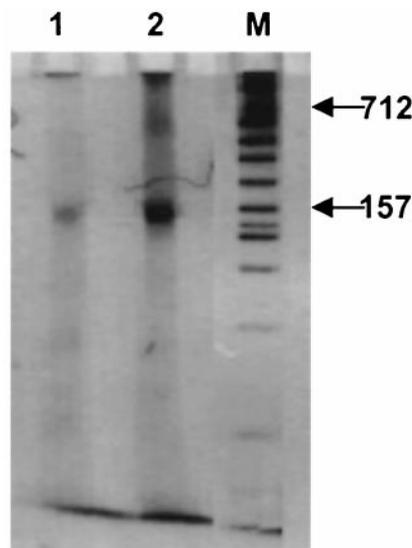


Fig. 3. Polyacrylamide gel electrophoresis of cell-free DNA isolated from human urine.

Urine samples were centrifuged for 10 min at 800g. DNA was isolated from the supernatants and subjected to agarose gel electrophoresis as described in *Materials and Methods*. Lanes 1 and 2, DNA from the urine of two volunteers. Lane 3, molecular weight markers.

**Blood transfusion.** Patients were women from the Surgery Department of the Cancer Research Center who, for medical reasons, received a blood transfusion 10–14 days previously. Urine samples were obtained from those patients who were transfused with blood from male donors. In this proof-of-principle study, male-specific DNA sequences were detected in five of nine urine samples obtained from women who were transfused with male blood (Fig. 4).

**Detection of fetal DNA.** A group of women in the first trimester of pregnancy were analyzed. Urinary DNA and fetal tissues were obtained from pregnant women attending an abortion clinic. Urinary DNA was tested as described in *Materials and Methods*. Fetal sex was determined by PCR of DNA isolated from tissue samples. Because a

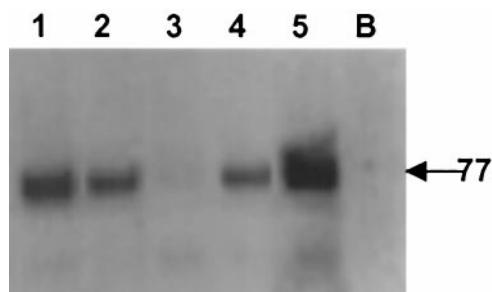


Fig. 4. Detection of Y-chromosome-specific sequences in the urine of women who had been transfused with blood from male donors.

Lanes 1–4, blood transfusion from male donors (10 days after transfusion of 250 mL of blood); lane 5, positive control (1 pg of male lymphocyte DNA); lane 6, blank (salt solution passed through all DNA isolation and analysis procedures).

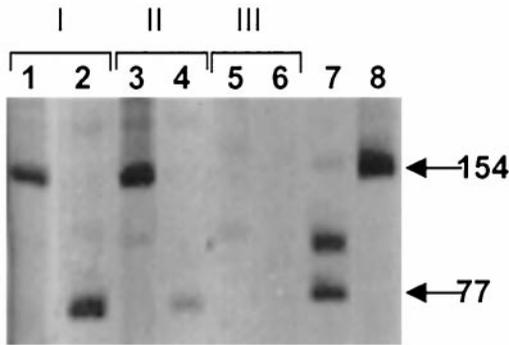


Fig. 5. Detection of Y-chromosome-specific sequences in the urine of women pregnant with male fetuses.

DNA was isolated from urine of women in the first trimester of pregnancy or from aborted material and analyzed as described in *Materials and Methods*. Lanes 1, 3, and 5, regular PCR analysis of fetal tissue DNA; lanes 2, 4, and 6, results of nested PCR of urinary DNA; lanes 7 and 8, controls for nested and regular PCR, respectively, performed with male lymphocyte DNA. I, II, and III indicate different patients.

large amount of fetal DNA was available, it was not necessary to perform nested PCR, and only Y1 and Y2 primers were used to amplify the 154-bp band. Fig. 5 shows the results of one experiment. Y-Chromosome-specific sequences were detected in the urine of women pregnant with male (patients I and II) but not female (patient III) fetuses. In 8 of 10 cases involving pregnancy with a male fetus at gestational age of 7–10 weeks, the Y-specific sequence was detected. All nine controls, women pregnant with female fetuses, gave negative results.

**Detection of K-ras mutations.** Two groups of patients were analyzed for K-ras mutations in their urinary DNA. It is known that 80–90% of pancreatic carcinomas bear K-ras mutations. We investigated the DNA in the urine of eight patients with pancreatic cancer (stage IV). Typical results are presented in Fig. 6. K-ras mutations were detected in five of eight urine samples from these patients. Unfortunately, in these experiments, we had no tumor tissue to verify the presence in tumor cells of the same K-ras mutations.

The second group consisted of seven colorectal cancer patients with advanced disease (stages III–IV). Urine samples were taken 24 h before surgery, and tissue

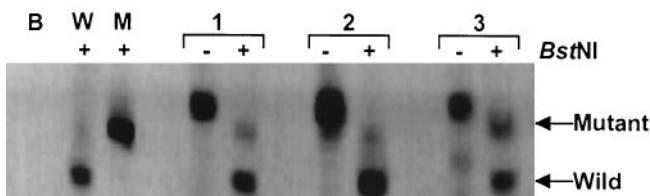


Fig. 6. Detection of K-ras mutations in urinary DNA from patients with pancreatic carcinomas.

DNA was isolated from urine of patients with pancreatic carcinomas, and the presence of K-ras mutations was analyzed as described in *Materials and Methods*. Numbers indicate different patients; W and M, controls performed with plasmids that contained wild-type and mutant K-ras, respectively; B, blank (salt solution passed through all DNA isolation and analysis procedures).

samples, tumor, and surrounding normal tissue were obtained during surgery. Thus, three DNA samples obtained from each patient, from the tumor, normal tissue, and urine, were analyzed (Fig. 7). K-ras mutations were detected in five of seven tumors. In four of five patients with tumor K-ras mutations, the same mutations were also detected in the urine samples. Two patients with no mutations in their tumors as well as nine healthy volunteers did not have K-ras mutations in their urinary DNA.

## Discussion

The results obtained demonstrate that the kidney barrier is, at least partially, permeable to polymeric DNA, particularly fragmented DNA from apoptotic cells. The molecular weight of DNA isolated from urine is high enough to allow its use for genetic analysis by traditional methods such as PCR and hybridization. In this proof-of-principle study, male-specific DNA sequences were detected in 5 of 9 urine samples obtained from women who were transfused with male blood and in 8 of 10 urine samples obtained from women bearing male fetuses. K-ras mutations were found in five of eight urine samples obtained from patients with pancreatic cancer as well as in the urine of four of five patients with colorectal adenocarcinomas who have corresponding mutations in their tumors. In the future, sensitivity can be improved by using larger volumes of urine and by applying more advanced methods, e.g., the use of labeled DNA primers in PCR or hybridization of PCR products with a specific labeled probe. Of course, enhancement of the sensitivity of any technique usually leads to new problems. In the case of PCR-based analysis, depending on the method used for product detection, these potential problems may include the appearance of nonspecific bands, higher background, or manifestation of low-level contamination with PCR products.

The DNA concentrations in the urine (2–96  $\mu\text{g/L}$ ) and

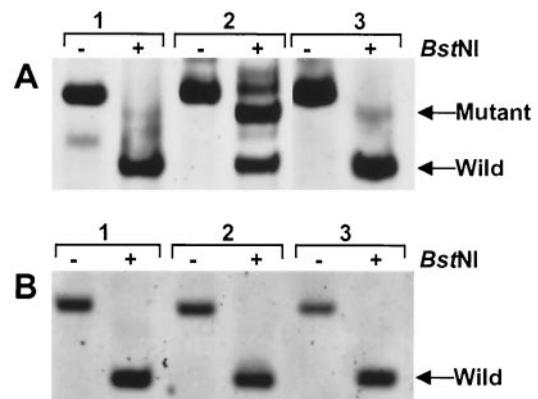


Fig. 7. Detection of K-ras mutations in urinary DNA from patients with colorectal carcinomas.

DNA was isolated as described in *Materials and Methods*: 1, DNA isolated from "normal" tissue; 2, DNA isolated from tumor tissue; 3, DNA isolated from urine. (A), patient with K-ras mutation in tumor tissue; (B), patient without K-ras mutation in tumor tissue.

plasma (1, 5) of healthy volunteers are similar. At first glance it looks strange. However, an analysis of the DNA half-life in plasma performed by Lo et al. (26) revealed that the turnover of cell-free DNA is very rapid. The mean half-life for fetal DNA was 16.3 min. In other words, the amount of DNA that passes through 1 mL of blood per day is much higher than the amount of DNA that is accumulated during this period in urine. A very rough estimate based on the data presented above indicates that ~0.5–2% of the free DNA that passes through the bloodstream crosses the kidney barrier and is excreted in the urine. The uncertainty of these values is also increased by the fact that a portion of cell-free DNA in the blood can originate from white blood cells destroyed during blood sampling and subsequent treatment. For example, the concentration of DNA in serum is much higher than in plasma from the same patient (15). Similarly, some of the DNA in urine can originate from cells that die in the urinary system and do not cross the kidney barrier. Therefore, more correct calculations should be based on analysis of specific sequences that are different from the bulk DNA in a body, such as fetus-specific or tumor-specific DNAs. Nevertheless, the amount of DNA that is excreted in urine is much higher than one can expect based on data from protein filtration studies in the kidney. This raises the question of the mechanism of DNA crossing this barrier. Mechanisms by which DNA crosses lipid bilayers have been proposed recently (27), but more research is needed to clarify the question.

Despite the questions raised by this study, the utility of these findings for genetic analysis is obvious. They demonstrate that DNA from dying cells can cross both the placental and kidney barriers and that specific sequences can be detected in urine by PCR.

We believe that further improvement of this methodology can lead to a significant increase in the sensitivity and reliability of DNA testing using urine. Several potential applications of this technology are similar to those expected from the analysis of plasma DNA (14, 26): (a) a simple and inexpensive method of prenatal sex determination at early gestational ages when ultrasound examination is not feasible; (b) prenatal detection of inherited genetic abnormalities as well as Rh incompatibility; (c) detection and monitoring of tumor growth; (d) evaluation of the effectiveness of tumor chemotherapy or radiation therapy; and (e) monitoring of rejection episodes after cell, tissue, or organ transplantation. With regard to monitoring graft rejection episodes, data published recently by Zhang et al. (28) should be mentioned. These authors reported detection of Y-chromosome-specific sequences of the *SRY* gene in cell-free urine samples from female patients who had received renal transplants from male donors but not in the urine of female patients who had received transplants from female donors. They concluded that the measurement of urinary DNA might be useful for monitoring of graft rejection. However, it is not clear whether the DNA sequences analyzed had crossed the

kidney barrier because transplanted organs belong to the urinary system.

There are some evident advantages of urinary DNA-based technology compared with analysis of plasma DNA: (a) urine-based tests are absolutely noninvasive; (b) urine is noninfectious for HIV and less infectious for many other pathogens; (c) the concentrations of DNA in plasma and urine are similar, but much more urine can be easily obtained for analysis; (d) isolation of DNA from urine is technically much easier because the protein concentration is >1000-fold lower; (e) in our study, we had no problem with PCR inhibitors, a well-known problem for DNA isolated from blood. In some cases, parallel analysis of urine and plasma DNA will increase the test reliability.

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