25. T. Tsuri *et al.*, *J. Med. Chem.* **40**, 3504 (1997).
26. Statistical significance was evaluated by one-way analysis of variance followed by Student's *t* test for unpaired values.

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Facile Detection of Mitochondrial DNA Mutations in Tumors and Bodily Fluids

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Examination of human bladder, head and neck, and lung primary tumors revealed a high frequency of mitochondrial DNA (mtDNA) mutations. The majority of these somatic mutations were homoplasmic in nature, indicating that the mutant mtDNA became dominant in tumor cells. The mutated mtDNA was readily detectable in paired bodily fluids from each type of cancer and was 19 to 220 times as abundant as mutated nuclear p53 DNA. By virtue of their clonal nature and high copy number, mitochondrial mutations may provide a powerful molecular marker for noninvasive detection of cancer.

The human mitochondrial (mt) genome is small (16.5 kb) and encodes 13 respiratory chain subunits, 22 transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs). Mitochondrial DNA is present at extremely high levels (10^3 to 10^4 copies per cell), and the vast majority of these copies are identical (homoplasmic) at birth (*1*). Expression of the entire complement of mt genes is required to maintain proper function of the organelle, suggesting that even slight alterations in DNA sequences could have profound effects (*2*). It is generally accepted that mtDNA mutations are generated during oxidative phosphorylation through pathways involving reactive oxygen species (ROS). These mutations may accumulate in part because mitochondria lack protective histones and the highly efficient DNA repair mechanisms that are seen in the nucleus (*3*).

Recently, several mtDNA mutations were found specifically in human colorectal cancer (*4*). To determine whether mt mutations could be identified in other cancer types, we studied primary bladder (n = 14), head and neck (n = 13), and lung (n = 14) tumors (*5*). Eighty percent of the mt genome of all the primary tumor samples was polymerase chain reaction (PCR) amplified (*6*) and sequenced manually (Fig. 1). Tumor mtDNA was compared with mtDNA from paired blood samples in all cases and mtDNA from corresponding normal tissue when available (*7*). Of the 292 sequence variants detected, 196 were previously recorded polymorphisms (*2, 8*), whereas 57 were previously unknown polymorphisms (Web table 1) (*9*). The remaining 39 variants were acquired (somatic) mutations identified in 64% (9 of 14) of the bladder cancer patients, 46% (6 of 13) of the head and neck cancer patients, and 43% (6 of 14) of the lung cancer patients (Table 1). Most of these mutations were T-to-C and G-to-A base transitions, indicating possible exposure to ROS-derived mutagens (*10*). Similar to the previous observation by Polyak *et al.* (*4*), the majority of the somatic mutations identified here were also homoplasmic in nature. In addition, several of the bladder and head and neck cancers studied here (Table 1) had multiple mutations, implying possible accumulation of mtDNA damage.

In the bladder tumors, mutation hot spots were primarily in the NADH (reduced form of nicotinamide adenine dinucleotide) dehydrogenase subunit 4 (*ND4*) gene (35%) and in the displacement-loop (D-loop) region (30%). The D-loop region is a critical site for both replication and expression of the mt genome because it contains the leading-strand origin of replication and the major promoters for transcription (*11*). Many (73%) of the mutations identified within protein-coding regions were silent, except for a (Val → Ala) substitution in the NADH dehydrogenase subunit 3 *(ND3)* and a seven–amino acid deletion in cytochrome b (*Cyt b*). The D-loop region was also commonly mutated in the head and neck cancer patients (67%). Two of the head and neck tumors (22%) contained mutations in the *ND4* gene at nucleotide pairs (nps) 10,822 and 11,150, resulting in amino acid substitution of Thr → Met and Ala → Thr, respectively. A similar tendency was observed in the lung cancer patients, demonstrating a high concentration of mutations in the D-loop region (70%).

We hypothesized that the homoplasmic nature of these mutations would make them readily detectable in paired bodily fluids. To test this, we extracted and directly amplified mtDNA from urine samples from patients diagnosed with bladder cancer. All three corresponding urine samples available in this study contained the mutant mtDNA derived from tumor tissues. For example, the mtDNA from a urine sample from bladder cancer patient 799 showed the same nucleotide transition (G → A) as seen in the tumor (Fig. 2A). In all cases, the urine sample contained a relatively pure population of tumor-derived mtDNA, comparable to that of the microdissected tumor sample. Consistent with this observation, saliva samples obtained from head and neck cancer patients contained no detectable wild-type signals (Fig. 2, B and C). By sequence analysis alone, we were able to detect mtDNA mutations in 67% (6 of 9) of saliva samples from head and neck cancer patients. In lung cancer cases, we were initially unable to identify mutant bands from paired bronchoalveolar lavage (BAL) fluids because of the substantial dilution of neoplastic cells in BAL fluid (*12*) (Fig. 2D). Thus, we applied a more sensitive oligonucleotide-mismatch ligation assay to detect mutated mtDNA. As shown in Fig. 3, both lung cancer mutations (arrows) were confirmed in tumor mtDNA with more dilute signals in the corresponding BAL samples and no signal in the corresponding normal

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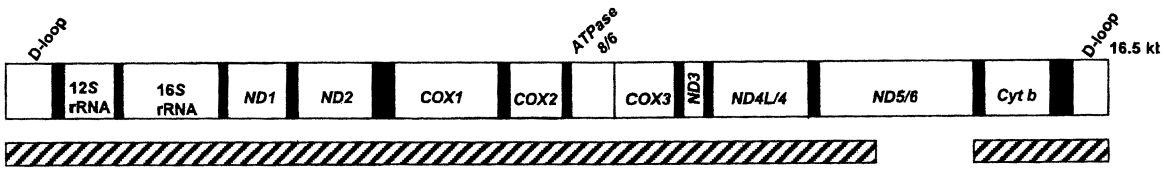

Fig. 1. Schematic representation of a linearized mt genome. Hatched bars indicate the regions sequenced in this study, and solid bars indicate the positions of tRNAs. rRNA, ribosomal RNA; *ND*, NADH dehydrogenase; *COX*, cytochrome c oxidase; *Cyt b*, cytochrome b; *ATPase*, ATP synthase.

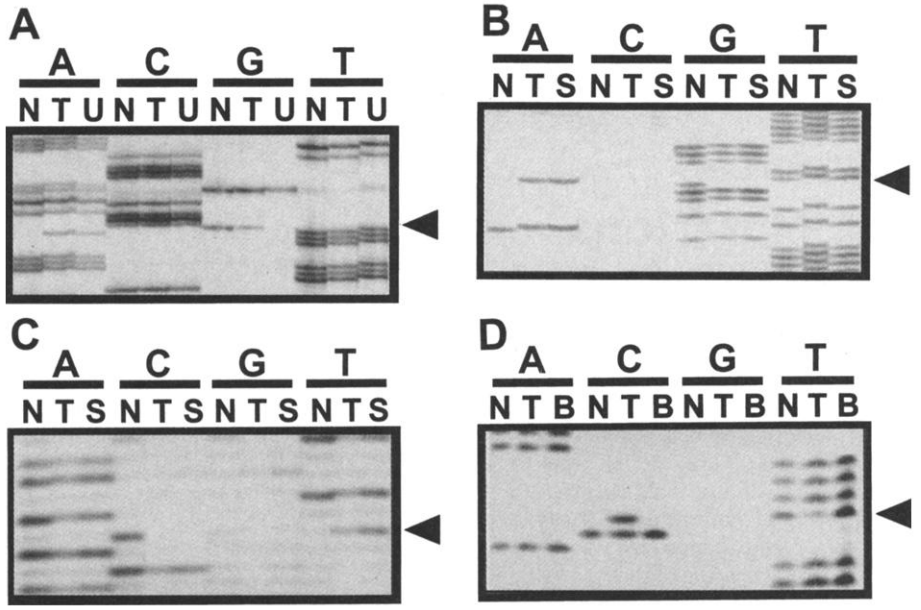

Fig. 2. Sequence detection of mutated mtDNAs in samples from tumors and bodily fluids. (**A**) The mt mutation was analyzed by direct sequencing of the tumor (T), normal (N), and corresponding urine (U) DNAs of bladder cancer patient 799. A single nucleotide change (G → A) at 2056 nps in the 16*S* rRNA gene is indicated. (**B** and **C**) Examples of somatic mutations in head and neck cancers. Both mutations at 16,172 nps (B) and 10,822 nps (C) were detected from saliva (S) samples from patients 1680 and 1708, respectively. (**D**) Mutated mtDNA at 2664 nps was not detected by sequence analysis in the paired BAL fluid (B), obtained from lung cancer patient 898. Arrows indicate the mutant band(s) in the appropriate lane.

tissues. Again, we detected the majority of mtDNA mutations (8 of 10) in BAL fluids, with the exception of two cases where the ligation assays were not feasible because of the sequence compositions (16,183 and 302 nps) adjacent to the mutations.

To quantitate this neoplastic DNA enrichment, we compared the abundance of mt gene mutations with that of nuclear-encoded p53 mutations in bodily fluids using a quantitative plaque assay. Nuclear and mt fragments that contained a mutated sequence were PCR amplified and cloned for plaque hybridization (*13*). Two BAL samples from lung cancer patients were chosen for analysis because they had mutations in both the mt and nuclear genomes. For p53 mutations, the percentages of neoplastic cells among normal cells for patients 1113 and 1140 were 0.1 and 3.0, respectively. Remarkably, the abundance of the corresponding mutated mtDNA (MT) was 22 and 52% when compared with the wild-type mt sequence (Fig. 4). This enrichment of mtDNA is presumably due to the homoplasmic nature of these mutations and the high copy number of mt genomes in cancer cells. Enrichment was further suggested by our observations with paraffin samples of head and neck cancer, where we were able to PCR amplify 2- to 3-kb fragments of mtDNA, but not nuclear p53 gene fragments of over 300 base pairs.

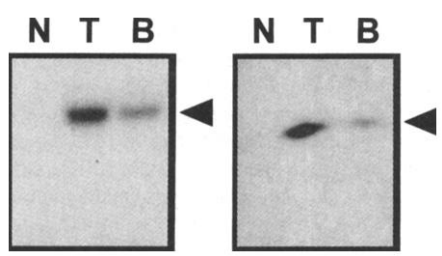

Fig. 3. Oligonucleotide-mismatch ligation assay (*23*) to detect mtDNA mutations in BAL. The arrows identify mutated mt sequences at 12,345 nps within tRNA (left) and at 2664 nps (right) within 16*S* rRNA in the tumor (T) DNA. More dilute signals are seen in the corresponding BAL (B) samples with no detectable signal from the paired normal (N) tissue.

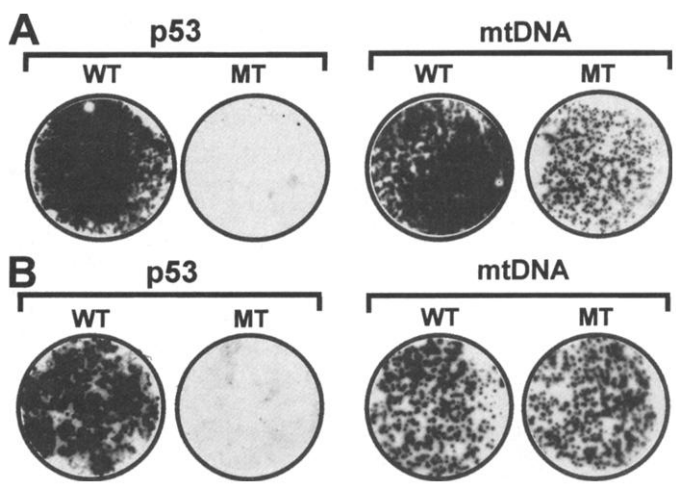

Fig. 4. Highly enriched mutated mtDNA in BAL samples from lung cancer patients. (**A**) Oligo-specific hybridization detected ~2000 plaques containing wild-type (WT) p53 clones in the BAL from patient 1113, and only two plaques (2 of 2000 = 0.1%) with the p53 gene mutation (left) were found in the primary tumor. The same BAL sample demonstrated a much greater enrichment of mutated mtDNA; 445 plaques contained mtDNA mutations (right) at 16,159 nps (445 of 2000 = 22.3%; 220-fold) compared with about 1500 wild-type clones. (**B**) A similar enrichment was seen in patient 1140, where oligo-specific hybridization detected 12 p53 mutant plaques among 437 wild-type clones (2.7 %) (left), whereas mutant mtDNA at 16,380 nps (right) represented over 50% of the plaques (52.3%, 460 of 880; 19-fold) amplified from mtDNA.

A role for mitochondria in tumorigenesis was hypothesized when tumor cells were found to have an impaired respiratory system and high glycolytic activity (*14, 15*). Recent findings elucidating the role of mitochondria in apoptosis (*16*) and the high incidence of mtDNA mutations in colon cancer (*4*) further support this hypothesis. Although additional investigation is needed to define the functional importance of mt mutations, our data establish that these mutations are frequent and present at high levels in all of the tumor types examined.

The homoplasmic nature of the mutated mitochondria remains puzzling. It is estimated that each cell contains several hundred to thousands of mitochondria and that each mitochondrion contains 1 to 10 genomes (*17*). Conceivably, certain mutated mtDNAs may gain a substantial replicative advantage. For example, mutations in the D-loop regulatory region might alter the rate of DNA replication by modifying the binding affinity of important trans-acting factors. Mitochondria that undergo the most rapid replication are likely to acquire more DNA damage, leading to an accumulation of mutational events. Although the mechanism may vary for other mutations (such as silent mutations in the *ND4* gene), the accumulation of a particular mtDNA mutation may become more apparent during neoplastic transformation. Even subtle mtDNA mutations may also gain substantial replicative advantage, perhaps through interactions with important nuclear factors. Homoplasmic transformation of mtDNA was observed in small populations of cells in other nonneoplastic but diseased tissues (*18*), sometimes associated with aging (*19*). We hypothesize that, in contrast to classic clonal expansion, the process may occur as "pseudoclonal" selection where stochastic segregation of mitochondria (*17*) together

Table 1. Summary of mtDNA mutations in primary tumors. Only the D-loop region was analyzed for lung cancer patients 1113, 1140, and 1174. N, sequence obtained from a normal sample; T, mutated sequence in tumor; aa, amino acid; del, deletion; ins, insertion. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; F, Phe; H, His; L, Leu; M, Met; T, Thr; and V, Val.

Patient number	Nucleotide position	Gene	DNA (N → T)	Protein
		Bladder cancer (9 of 14, 64%)		
1124	114	D-loop	T → C	–
580	302	D-loop	del C	–
580	386	D-loop	C → A	–
799	2056*	16S rRNA	G → A	–
716	2445	16S rRNA	T → C	–
1127	3054*	16S rRNA	G → A	–
884	10,071	*ND3*	T → C	L → L
884	10,321	*ND3*	T → C	V → A
884	10,792	*ND4*	A → G	L → L
884	10,793	*ND4*	C → T	L → L
899	10,822	*ND4*	C → T	H → H
716	10,978	*ND4*	A → G	L → L
870	11,065	*ND4*	A → G	L → L
870	11,518	*ND4*	G → A	L → L
884	12,049	*ND4*	C → T	F → F
874	12,519	*ND5*	T → C	V → V
580	15,642	*Cyt b*	del	7 aa
899	16,189	D-loop	ins T	–
1124	16,265	D-loop	A → C	–
1127	16,532*	D-loop	A → T	–
		Head and neck cancer (6 of 13, 46%)		
1637	75	D-loop	G → A	–
1680	302	D-loop	del C	–
1565	514	D-loop	Ins CG	–
1684	1811	16S rRNA	A → G	–
1708	10,822	*ND 4*	C → T	T → M
1678	11,150	*ND 4*	G → A	A → T
1680	16,172	D-loop	C → T	–
1680	16,292	D-loop	C → T	–
1680	16,300	D-loop	A → G	–
		Lung cancer (6 of 14, 43%)		
1174	150	D-loop	C → T	–
1174	195	D-loop	T → C	–
902	302	D-loop	del C	–
898	2664	16S rRNA	T → C	–
915	5521	tRNA Trp	G → A	–
915	12,345	tRNA Leu	G → A	–
915	16,183	D-loop	C → A	–
915	16,187	D-loop	C → T	–
1113	16,519	D-loop	T → C	–
1140	16,380	D-loop	G → A	–

*Heteroplasmic mutation.

with neoplastic clonal expansion driven by nuclear mutations leads to a homogeneous population of a previously "altered" mitochondrion (Web figure 1) (*9*).

The large number of mt polymorphisms identified here and elsewhere (*2*) likely reflects the high mutation rate of mtDNA, which is thought to be caused mainly by high levels of ROS (*20*). In agreement with this, our data imply that constitutive hypervariable areas such as the D-loop region represent somatic mutational hot spots. As further mutations are tabulated in primary tumors, DNA-chip technology can be harnessed to develop high-throughput analyses with sufficient sensitivity to detect these mutations in most bodily fluids (*21*, *22*). Because of its high copy number, mtDNA may provide a distinct advantage over other nuclear genome–based methods for cancer detection.

References and Notes

1. R. N. Lightowlers, P. F. Chinnery, D. M. Turnbull, N. Howell, *Trends Genet.* **13**, 450 (1997).
2. MITOMAP: A Human Mitochondrial Genome Database, Center for Molecular Medicine, Emory University, Atlanta, GA (www.gen.emory.edu/mitomap.html).
3. D. L. Croteau and V. A. Bohr, *J. Biol. Chem.* **272**, 25409 (1997).
4. K. Polyak *et al.*, *Nature Genet.* **20**, 291 (1998).
5. Paired normal and tumor specimens along with blood and bodily fluids were collected after surgical resections with prior consent from patients in the Johns Hopkins University Hospital. Tumor specimens were frozen and microdissected on a cryostat so that the tumor samples contained greater than 70% neoplastic cells. DNA from tumor sections was digested with 1% SDS/Proteinase K, extracted by phenol-chloroform, and ethanol precipitated. Control DNAs from peripheral lymphocytes, matched normal tissues, urine, saliva, and BAL fluid were processed in the same manner as described in (*12*).
6. Mitochondrial DNAs were amplified with overlapping primers (*4*) in PCR buffer containing 6% dimethyl sulfoxide. About 5 to 20 ng of genomic DNA was subjected to the step-down PCR protocol: 94°C for 30 s, 64°C for 1 min, 70°C for 3 min, three cycles; 94°C for 30 s, 61°C for 1 min, 70°C for 3 min, three cycles; 94°C for 30 s, 58°C for 1 min, 70°C for 3.5 min, 15 cycles; 94°C for 30 s, 57°C for 1 min, 70°C for 3.5 min, 15 cycles; and a final extension at 70°C for 5 min. PCR products were gel-purified with a Qiagen gel extraction kit (Qiagen), and sequence reactions were performed with Thermosequenase (Perkin-Elmer) with the cycle conditions (95°C for 30 s, 52°C for 1 min, and 70°C for 1 min for 25 cycles).
7. Corresponding normal tissues from four patients (874, 915, 1684, and 1678) were available, and DNA was extracted from paraffin samples as described previously (*10*).
8. R. M. Andrew *et al.*, *Nature Genet.* **23**, 147 (1999).
9. Supplementary material is available at *Science* Online (www.sciencemag.org/feature/data/1048413.shl).
10. J. Cadet, M. Berger, T. Douki, J. L. Ravanat, *Rev. Physiol. Biochem. Pharmacol.* **131**, 1 (1997).
11. J. W. Taanman, *Biochim. Biophys. Acta* **1410**, 103 (1999).
12. S. A. Ahrendt *et al.*, *J. Natl. Cancer Inst.* **91**, 332 (1999).
13. Subcloning of PCR fragments into phage vector was performed according to the manufacturer's instructions (Stratagene). Titered plaques were plated and subjected to hybridization with tetramethylammonium chloride as a solvent. Positive signals were confirmed by secondary screenings. Oligonucleotides (oligos) used for this assay were as follows. For patient 1113, p53 and mtDNA sequence alterations were detected with oligos containing either wild-type (p53: 5′-GTATTTGGATGTCAGAAACACTT-3′; mtDNA: 5′-ACTTCAGGGTCATAAAGCC-3′) or MT (p53: 5′-GTATTTGGATGTCAGAAACACTT-3′; mtDNA: 5′-ACTTCAGGGCCATAAAGCC-3′) sequences, respectively. For patient 1140, oligos 5′-ACCCGCGTCCGCGCCATGGCC-3′ and 5′-ACCCGCGTCCTCGCCATGGCC-3′ were used to detect wild-type and MT sequences, respectively.
14. O. Warburg, *Science* **123**, 309 (1956).
15. J. W. Shay and H. Werbin, *Mutat. Res.* **186**, 149 (1987).
16. D. R. Green and J. C. Reed, *Science* **281**, 1309 (1998).
17. D. C. Wallace, *Annu. Rev. Biochem.* **61**, 1175 (1992).
18. ———, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8746 (1994).
19. K. Khrapko *et al.*, *Nucleic Acids Res.* **27**, 2434 (1999).
20. C. Richter, J. W. Park, B. N. Ames, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 6465 (1988).
21. M. Chee *et al.*, *Science* **274**, 610 (1996).
22. S. A. Ahrendt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7382 (1999).
23. Fragments containing mutations were PCR amplified and then ethanol precipitated. For each mutation, discriminating oligos that contained the mutated base at the 3′ end were designed (TAACCATA-3′ for patient 915 and TCTCTTACC-3′ for patient 898). Immediately adjacent [^{32}P] end-labeled 3′ sequences (5′-CACACTACTA-3′ for patient 915 and 5′-TTTAACCAG-3′ for patient 898) were used as substrate together with discriminating oligos for the ligation reaction. After a denaturing step of 95°C for 5′, the reactions were incubated for 1 hour at 37° in the presence of T4 DNA ligase (Life Technologies, Rockville, MD) in a buffer containing 50 mM tris-HCl, 10 mM $MgCl_2$, 150 mM NaCl, 1 mM Spermidine, 1 mM adenosine triphosphate (ATP), and 5 mM dithiothreitol and analyzed on denatured 12% polyacrylamide gels [J. Jen *et al.*, *Cancer Res.* **54**, 5523 (1994)].

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