[CANCER RESEARCH 61, 1299–1304, February 15, 2001]

Advances in Brief

Detection of Mitochondrial DNA Mutations in Pancreatic Cancer Offers a "Mass"-ive Advantage over Detection of Nuclear DNA Mutations[1]

Jessa B. Jones, Jason J. Song, Paula M. Hempen, Giovanni Parmigiani, Ralph H. Hruban, and Scott E. Kern[2]

Predoctoral Program in Human Genetics [J. B. J.], Departments of Oncology [P. M. H., J. J. S., G. P., R. H. H., S. E. K.], Pathology [R. H. H., G. P., S. E. K.], and Biostatistics [G. P.], Johns Hopkins University, Baltimore, Maryland 21231

Abstract

We sequenced the complete 16.5-kb mitochondrial genome (mtDNA) in 15 pancreatic cancer cell lines and xenografts. Homoplasmic mtDNA somatic mutations and novel variants were identified in nearly all samples. Southern blot analysis and direct sequencing of mutation sites showed that the intracellular mass of mtDNA was greatly (6–8-fold) increased in pancreatic cancer cells in relation to corresponding normal cells; this property accounted for and greatly facilitated the identification of these mutations among the dense desmoplastic host reaction characteristic of primary pancreatic cancers. Structural characteristics and mathematical modeling of the evolution of mtDNA mutations suggested that many of the mutations identified might represent a random evolution of homoplasmic variants, rather than necessarily being a product of selective pressures. Complete sequencing of the nuclear *MnSOD* gene, which protects cells from the mitogenic and toxic effects of oxygen radicals, did not reveal any mutations. Nevertheless, the nearly ubiquitous prevalence and high copy number of mtDNA mutations suggest that they be considered of promising clinical utility in diagnostic applications.

Introduction

Hundreds to thousands of copies of the 16.5-kb human mitochondrial genome are present within every human cell (1). This circular genome encodes 13 polypeptides comprising various subunits of the respiratory chain complexes, as well as 22 tRNAs and two rRNAs[3] used in mitochondrial protein synthesis. The close proximity of the mtDNA to ROS generated by oxidative phosphorylation, when combined with a low efficiency of mtDNA repair systems, produce a mutation rate of mtDNA that is 10 times higher than nDNA (2).

The biology of the mitochondria suggests that its genome may be an attractive target for mutations that could drive tumorigenesis. Mitochondria generate ROS, which at slightly elevated concentrations are highly mitogenic molecules that are also mutagenic to the nuclear and mitochondrial genomes (3). Mitochondria are also known to play an important role in regulating or effecting apoptosis (reviewed in Ref. 4). Furthermore, it has long been known that mitochondria of rare oncocytic tumors can accumulate to a high density (5), and more commonly, that tumors tend to up-regulate glycolysis, in turn leading to a lesser dependence on the mitochondria for oxidative phosphorylation (6).

The "mitochondrial bottleneck" is the presumptive process by which the maternal oocyte contributes a homogeneous population of mtDNA genomes to the zygote, resulting in an organism with only one mitochondrial genotype, *i.e.*, homoplasmy (7). Recently, however, several groups have identified homoplasmic variants and somatic mutations of mtDNA in various tumor types (8–10). It has proven empirically easier to detect known mtDNA mutations in clinical exfoliative samples of cancer patients as compared with detection of mutations of nuclear genes, although the physical basis of this observation remained unexplored. In the current study, we investigate the mtDNA mutations of pancreatic cancer to address the evidence of a role in tumorigenesis and discuss the physical basis, benefits, and limitations of mtDNA analysis as a diagnostic tool.

Materials and Methods

Samples. Adenocarcinomas of the pancreas and distal common bile duct resected at The Johns Hopkins Hospital between 1992 and 1997 were xenografted as has been described, with normal duodenal tissue from the same patients also frozen for further study (11). The pancreatic cancer cell lines AsPc1, BxPc3, CAPAN1, CAPAN2, Hs766T, Su86.86, CFPAC1, MiaPaCa 2, and Panc1 were purchased from American Type Culture Collection (Manassas, VA). Pancreatic cancer cell line COLO357 was obtained from the European Collection of Animal Cell Cultures (Salisbury, United Kingdom).

***MnSOD* Sequencing.** A panel of 100 pancreatic cancer xenografts was analyzed by PCR for the presence of homozygous deletions of the *MnSOD* locus using gene-specific PCR primers. This panel was evaluated for LOH at 6q25.2 using polymorphic dinucleotide repeat markers *D6S1579*, *D6S1581*, *D6S305*, *D6S1599*, and *D6S1719* (Research Genetics, Huntsville, AL). In those samples exhibiting LOH, all five exons were amplified using intronic or 5′ untranslated region primers and were directly sequenced to examine the complete coding regions. PCR primers and sequencing primers used in this project are available upon request.

Complete Mitochondrial Genome Sequencing. The entire 16.5-kb mitochondrial genome was amplified and sequenced in samples from five xenografted primary pancreatic adenocarcinomas and the cell lines AsPc1, BxPc3, CAPAN1, CAPAN2, COLO357, Hs766T, Su86.86, CFPAC1, MiaPaCa 2, and Panc1. Total cellular DNA was prepared for each sample as described (12). To avoid amplification of nuclear mitochondrial pseudogenes, the mitochondrial genome was amplified as 10 overlapping 1–3-kb PCR products (9). Direct automated sequencing of amplified templates was performed on Beckman CEQ2000 and ABI377 machines. Resulting sequence data files were analyzed and compared with the standard Anderson mtDNA sequence (13) using the Sequencher analysis program (Gene Codes, Ann Arbor, MI). Homoplasmic deviations from the standard sequence were compared with the on-line Mitomap database of previously published mtDNA mutations and polymorphisms.[4] Confirmation of those mutations that were not described previously as polymorphisms was performed by sequencing in the reverse direction on independent PCR products. To determine which mutations were somatic, DNA samples from normal nonneoplastic tissues corresponding to each tumor were sequenced as above. Limiting dilution PCR, followed by allele-specific oligonucleotide hybridization, was used to detect the potential presence of low levels of mutant mtDNA in normal tissue. This (digital PCR) was performed similarly to methods described but used sequence-specific dot blot hybridization for allele discrimination (14).

Received 10/30/00; accepted 12/29/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported by the NIH Specialized Program of Research Excellence in Gastrointestinal Cancer Grant CA 62924.

[2] To whom requests for reprints should be addressed, at The Johns Hopkins University, 451 CRB, Baltimore, MD 21231. Phone: (410) 614-3314; E-mail: sk@jhmi.edu.

[3] The abbreviations used are: rRNA, ribosomal RNA; mtDNA, mitochondrial DNA; nDNA, nuclear DNA; ROS, reactive oxygen species; LOH, loss of heterozygosity; MnSOD, manganese superoxide dismutase.

[4] MITOMAP: A Human Mitochondrial Genome Database; see Internet address http://www.gen.emory.edu/mitomap.html.

mtDNA Mass Comparison. To determine the relative copy number (mass) of mtDNA upon comparison of two sets of paired pancreatic cancer xenografts and surrounding normal tissue, Southern blot hybridizations were performed. To detect mtDNA, a 1-kb probe from the mitochondrial *mtND1* gene was labeled by random primer method with [^{32}P]dCTP and applied to blots of 10 μg of *Eco*RI-digested cellular DNAs using standard protocols. Total nDNA was relatively quantitated by reprobing the same blot with a random primer-labeled *Cot*I DNA probe (Bio-Rad, Hercules, CA). The relative amounts of both mtDNA and nDNA were estimated by PhosphorImager and image analysis densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Detection of mtDNA Mutation *versus* nDNA Mutations. Two DNA sets, each consisting of a primary pancreatic tumor (C16 and C19), a xenograft of the tumor (PX16 and PX19), and corresponding normal duodenum (N16 and N19) from two patients with known mtDNA and nDNA (K-*ras*) mutations, were obtained. The neoplastic cellularity of each sample was estimated by light microscopy. Both primary cancer samples consisted of 30% or fewer neoplastic cells. Amplified products containing each of the known mutations were created using 40 ng of template DNA for each sample. The amplified DNAs were directly sequenced.

Bioinformatics. tRNA structure prediction was performed using the tRNAscan-SE program available on-line (Washington University, St. Louis, MO). Sequence alignments were performed using public resources (National Center for Biotechnology Information).

Mathematical Modeling. To address the possibility that homoplasmic mutations of mtDNA may arise in the absence of selection, we created a simulation algorithm to statistically model mtDNA segregation.[5] The algorithm probabilistically generates cell histories under a variety of conditions. It is written in the publicly available software package R and is available for download.[6] On the basis of current literature, we started with cells containing an average of 100 mitochondria, each containing one genome (15). In this model, a single mtDNA genome/cell is mutated at one position at time zero. The fate of the mutant mtDNA species is then evaluated after the parent cell completes 2000 cell division events. At each cell division, a percentage (p) of mitochondria receive a signal to divide. In our modeling experiment, p was set to 0.05, 0.5, or 0.95. The parent cell divides when a sufficient number of new mitochondria (at least twice the original number) are present. The distribution of mitochondria to the two daughter cells is random.

Results

Complete Mitochondrial Genome Sequencing. Among the 10 pancreatic cancer cell lines sequenced, 226 homoplasmic variations from the standard Anderson mtDNA sequence were identified. Of this group, 49 variants did not match previously reported polymorphisms (Table 1). Of these 49 variants, 26 were silent changes within expressed sequences. The 23 remaining homoplasmic mutations within coding sequences, or regulatory sequences, exhibit potential functional significance.

Seven of these 23 mutations were in mitochondrial rRNA genes; seven mutations occurred in mitochondrial NADH dehydrogenase genes coding for complex I proteins (*mtND1, mtND2, mtND3, mtND4, mtND4L,* and *mtND5*), one was a mutation of a complex III mitochondrial cytochrome *c* oxidoreductase gene (*mtCytB*), four were mutations of complex IV mitochondrial cytochrome *c* oxidase genes (*mtCO1, mtCO$_2$,* and *mtCO3*), two were mutations of complex V mitochondrial ATP synthase genes (*mtATP6* and *mtATP8*), and two were mutations of the D-loop regulatory region of mtDNA (Table 1).

Two cell lines (AsPc1 and Su86.86) had no apparent functionally significant homoplasmic mutations, *i.e.*, all variants were previously described polymorphisms, whereas each of the remaining eight cell lines harbored at least one new variant of the coding sequence. Two cell lines (Hs766T and MiaPaCa 2) each contained only one new coding sequence mutation. Three other lines (Panc1, CAPAN2, and BxPc3) each contained two new coding mutations. CFPAC1 contained four new coding mutations, whereas CAPAN1 and COLO357 each, respectively, contained five and six individual homoplasmic potentially functional mutations not reported previously. There were no major deletions or rearrangements of mtDNA in any sample. All new mutations were single bp substitutions; nearly all were transition mutations consistent with damage from ROS (16). Each of the mutations of a protein coding sequence resulted in a single missense mutation. The functional significance of these mutations was unclear. However, one of the mutations (G9804A) of the *mtCO3* gene in cell line BxPc3 is reported as a secondary mutation in Leber hereditary optic neuropathy (17).

Unfortunately, because normal DNA corresponding to the commercial cancer cell lines was not available, it was not possible to differentiate those cell line mutations that arose somatically from those mutations that were rare constitutional variants. To search for somatic mutations, the complete mitochondrial genome of five xenografted pancreatic cancers was sequenced. One hundred twenty-seven homoplasmic variants were detected, of which 27 were not among the polymorphisms reported previously. Nearly all of these variants were also detected in the corresponding normal DNA of the patients.

Table 1 *New mtDNA variants observed in pancreatic cancer cell lines*

Cell lines	Nucleotide position	Gene	Mutation	Protein sequence change
MiaPaCa2	687	12S rRNA	G → A	NA[a]
CAPAN1	1243	12S rRNA	T → C	NA
CAPAN1	1406	12S rRNA	T → C	NA
COLO357	1676	16S rRNA	A → G	NA
Panc1	2015	16S rRNA	G → A	NA
COLO357	2222	16S rRNA	T → C	NA
CFPAC	2905	16S rRNA	A → G	NA
CAPAN1	3421	*ND1*	G → A	Val → Met
CAPAN1	3505	*ND1*	A → G	Thr → Ala
CFPAC	3654	*ND1*	C → T	Silent
COLO357	4580	*ND2*	G → A	Silent
CFPAC	4811	*ND2*	A → G	Silent
AsPc1	5918	*CO1*	T → C	Silent
COLO357	5973	*CO1*	G → A	Ala → Thr
CFPAC	5999	*CO1*	T → C	Silent
CFPAC	6047	*CO1*	A → G	Silent
CFPAC	6146	*CO1*	A → G	Silent
CAPAN2	6267	*CO1*	G → A	Ala → Thr
BxPc3	6869	*CO1*	C → T	Silent
CAPAN1	7151	*CO1*	C → T	Silent
Hs766T	7986	*CO2*	G → T	Arg → Gln
CFPAC	8696	ATP6	T → C	Met → Thr
CFPAC	9070	ATP6	T → G	Ser → Ala
AsPc1	9078	ATP6	T → C	Silent
COLO357	9254	*CO3*	A → G	Silent
COLO357	9368	*CO3*	A → G	Silent
BxPc3[b]	9804	*CO3*	G → A	Ala → Thr
CAPAN2	10176	*ND3*	G → A	Gly → Thr
CAPAN1	10715	*ND4L*	C → T	Silent
CFPAC	10970	*ND4*	T → C	Phe → Leu
CFPAC	11009	*ND4*	T → C	Silent
CAPAN2	11152	*ND4*	T → C	Silent
CAPAN1	11674	*ND4*	C → T	Silent
BxPc3	11703	*ND4*	T → C	Leu → Pro
COLO357	11781	*ND4*	T → C	Ile → Thr
CAPAN1	11974	*ND4*	A → G	Silent
CAPAN1	12414	*ND5*	T → C	Silent
CFPAC	12561	*ND5*	G → A	Silent
CFPAC	12717	*ND5*	C → T	Silent
MiaPaCa 2	12954	*ND5*	T → C	Silent
CAPAN2	13500	*ND5*	T → C	Silent
Panc1	14552	*ND6*	A → G	Val → Ala
Panc1	14650	*ND6*	C → T	Silent
CFPAC	14866	*CytB*	C → T	Silent
AsPc1	15646	*CytB*	C → T	Silent
CAPAN1	15784	*CytB*	T → C	Silent
CAPAN1	15884	*CytB*	G → C	Ala → Pro
COLO357	16527	D-Loop	C → T	NA
COLO357	16537	D-Loop	C → T	NA

[a] NA, not applicable.
[b] This mutation has been reported as a secondary mutation in Leber hereditary optic neuropathy.

[5] G. Parmigiani, J. Jones, and S. E. Kern, manuscript in preparation.
[6] Internet address: http://biosun01.biostat.jhsph.edu/~gparmigi/mito.html.

Table 2 *Somatic homoplasmic mutations of pancreatic cancer xenografts*[a]

Xenograft	Nucleotide position	Gene	Mutation	Protein sequence change
PX16	14603	*mtND6*	G → A	Ser → Phe
PX17	2805	16S rRNA	A → T	NA[b]
PX19	15983	tRNA-Pro	T → C	NA
PX27	3670[c]	*mtND1*	G → A	Ala → Thr

[a] PX24 contained no homoplasmic somatic mutations.
[b] NA, not applicable.
[c] This mutation was present in the heteroplasmic germ-line of this patient but was a homoplasmic mutant in the tumor.

However, four of the five xenografts were shown to harbor a single somatic homoplasmic mtDNA mutation (Table 2).

To date, the mitochondrial mutations seen in passaged tumors have been confirmed as originating in the patients' primary tumors. This confirmation is seen in both of our cases available for this comparison as well as in all cell lines examined in a prior study of mtDNA changes (9). Another study of mtDNA performed exclusively on primary tumors found mutational patterns similar to our study (8). Pancreatic cancers have been characterized extensively, with all sequence changes and allelic losses found in xenografts being confirmed in parallel-derived lines and primary tumors (11, 12).

Functional Significance of Somatic Mutations. Of the four somatic mutations discovered in the xenografted cancers, one mutation was an A-to-T substitution in the mitochondrial rRNA, of unknown significance. Two of the somatic mutations occurred in protein-coding regions of the mitochondrial genome, both coding for protein subunits of the mitochondrial respiratory chain NADH dehydrogenase complex, mtND1 and mtND6. The degree of evolutionary conservation of the affected residues was estimated using a multiple sequence alignment comprising 105 metazoan mitochondrial *ND1* and *ND6* sequences (data not shown). The mtND6 serine residue mutated by the transition at mtDNA position 14603 in tumor sample PX16 was conserved in 71% of metazoan mtDNAs. Mutation of this residue to phenylalanine may thus significantly affect the activity of this protein. The second mutation at position 3670 replaces an alanine codon in the *mtND1* gene with threonine. This alanine is conserved in 74% of metazoans but tolerates a threonine mutation in the Pacific oyster. The effect of this mutation on complex I activity remains unclear.

The fourth mutation is a C-to-T transition at position 15983 in the mitochondrial tRNA for proline. A predicted tRNA structure was created using tRNAscan (data not shown). From this structure, the effect of the mutation would be to change a wild-type U/A stem bp to a U/G bp. Although less stable than U/A pairing, U/G base pairing is often seen in wild-type tRNA structures.

Detection of Mutant mtDNA in Normal Tissue. The human mitochondrial genome has been sequenced extensively. An on-line database, Mitomap, lists the compilation of disease-causing mutations, as well as hundreds of mtDNA polymorphisms that have been observed[4] (24). Most (227 of 353; 64%) homoplasmic mtDNA variations observed in this study were present in this database as known mtDNA polymorphisms. Twenty-three (85%) of the 27 of the nonlisted homoplasmic variations from the Anderson sequence in our tumor xenografts were also detected in corresponding normal tissue. Thus, these variations represent undescribed rare polymorphisms of mtDNA, *i.e.*, individual constitutional variation. Each xenograft harbored from 2 to 10 of these new variants in homoplasmy.

In one sample, xenograft PX27, the homoplasmic G-to-A variation from the Anderson sequence at position 3670 was also detected by direct sequencing as coexisting with the consensus sequence in the corresponding normal duodenal tissue (Fig. 1*A* and Table 2). This indicates that during evolution of the tumor, a preexisting heteroplasmic population of mitotypes had evolved to apparent homoplasmy. The presence of mutant mtDNAs in corresponding normal tissues at a level below detection by direct sequencing had not yet, however, been excluded as a possibility in the other patients. Therefore, we studied the G-to-A mutation at position 14603 in PX16 by digital PCR. Of 191 single mtDNA template PCR products from the corresponding normal DNA, one product contained the mutant allele, whereas all

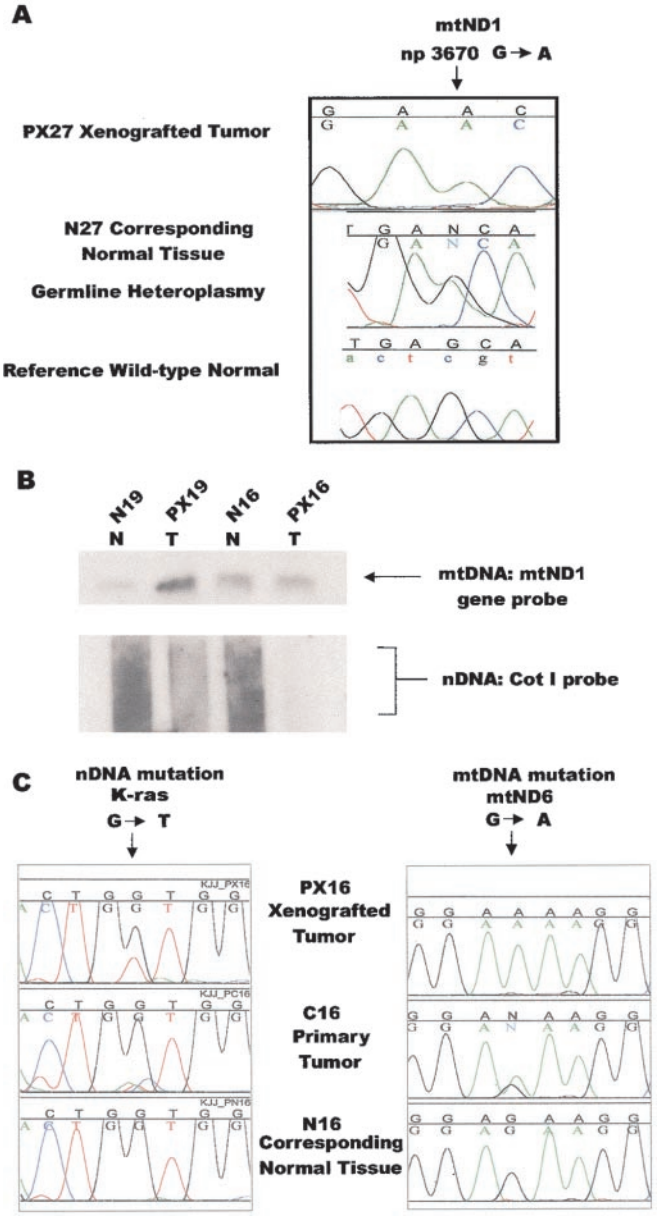

Fig. 1. mtDNA analysis. *A*, direct sequencing of pancreatic cancer xenograft PX27 revealed a homoplasmic coding sequence mutation in *mtND1*. *Arrow*, location of the mutation G-to-A at nucleotide position 3670. Normal DNA from the same patient (*N27*) has germ-line heteroplasmy for this mutation. The presence of both the wild-type *G* allele and the mutant *A* allele are detected. An example of the wild-type homoplasmic *G* allele found in all other samples is shown (*Reference Wild-type Normal*). *B*, Southern blot shows a relative increase in mtDNA mass in the cancer cells. Restriction-digested normal DNA (*N19* and *N16*) and tumor DNA (*PX19* and *PX16*) were probed with; *top*, mtDNA probe containing sequence from *mtND1*; and *bottom*, nDNA probe containing *Cot*I repetitive human DNA sequences. Phosphorimaging and densitometry of this blot indicate a 7.7-fold increase in intensity of mtDNA signal in PX19 relative to N19 and a 6-fold increase in intensity of mtDNA signal in PX16 relative to N16 when normalized to nDNA signal. *C*, cancer-specific mutations are best represented in mtDNA as compared with nuclear DNA. *Arrow* (both panels), nucleotide of interest. *Left*, in tumor xenograft PX16, both a wild-type *G* allele of K-*ras* and mutant *T* allele are detected. In primary tumor C16, only the wild-type *G* allele is reliably detected. In normal tissue from same patient, N16, only the wild-type *G* allele is present. *Right*, PX16 shows homoplasmy for the mtDNA mutation, *i.e.*, only mutant *A* allele appears. In the primary tumor C16, both the mutant *A* allele and wild-type *G* allele are detectable. In the normal tissue of this patient, N16, the wild-type *G* allele is present in homoplasmy. In all chromatograms, individual bases are identified by the following color code: *A, green*; *G, black*; *C, blue*; *T, red*.

others contained the wild-type allele (data not shown). This borderline result was felt to be inconclusive, consistent with a trivial explanation such as disease metastasis or surgical contamination during specimen acquisition, as well as with an extremely low level of constitutional heteroplasmy.

Statistical Modeling of Mutant mtDNA Segregation to Homoplasmy. A probabilistic modeling algorithm[5] was created to study the likelihood that the homoplasmic mtDNA mutations seen here and elsewhere may have evolved in the absence of selection. Recently, Cavlier *et al.* (15) determined a mean copy number in fibroblast cells of two mtDNAs per "mitochondrial particle" by flow cytometry and PCR. Through PCR genotyping of individual mtDNAs, they found that all of the mtDNA in the majority of these particles was of only one genotype, either all mutant or all wild-type. We interpreted this result to be consistent with a single unit of mtDNA per mitochondrion as the replicative template. Each iteration of the modeling algorithm followed the random segregation of mtDNA in a model cell with 100 mitochondria, each having one mtDNA, as it completed 2000 cell divisions (a general approximation of the number of cell divisions during tumorigenesis). One to 2% of the daughter cells at the end of the simulation contained homoplasmic mutant mtDNA. Furthermore, by decreasing the number of mitochondria/cell from 100 to 10, the generation of homoplasmic mutant mtDNA cells increased to 3% when 95% of the mitochondria in the cell received the signal to replicate each division. When the "permission to replicate" parameter was decreased to 50% of mitochondria, the percentage of homoplasmic mutant mtDNA containing daughter cells increased to 5% and further increased to 8% when the most stringent conditions (5% of mitochondria replicate) were applied.

Detection of mtDNA and nDNA Mutations in Primary Tumors. A Southern blot was performed to compare the levels of mtDNA in pancreatic cancer *versus* normal cells. DNA from two xenografted pancreatic tumors (PX16 and PX19) were compared with DNA samples of normal duodenum from the same patients (Fig. 1*B*). The quantity of mtDNA was 7.7-fold higher than normal in tumor sample PX19 and 6-fold higher than normal for sample PX16 when normalized for nDNA levels, as determined by PhosphorImager densitometry.

Two sets of samples (patients 16 and 19), each composed of a pancreatic primary cancer sample, a xenograft of this sample, and a corresponding normal sample from the same patient, were then studied by sequencing. Both mtDNA and nDNA (K-*ras*) mutations of these cancers were known. Xenografts PX16 and PX19 had the homoplasmic mtDNA mutations described above, while also harboring an nDNA G-to-T mutation in codon 12 of the K-*ras* gene. The malignant cellularity of both primary cancers was histologically estimated at 30% or less. PCR products containing the mutations were amplified from the primary cancer, xenograft, and normal samples for these two cases. The results of direct sequencing of these products are shown in Fig. 1*C*.

In the case of patient 16, the nuclear K-*ras* gene mutation was detectable on one allele in the xenograft sample, absent from the normal sample, and not visible in the primary cancer because of contamination by nonneoplastic cells (Fig. 1*C*). In striking contrast, the mutant *A* allele (*i.e.*, the mtDNA mutation) was clearly present in both the xenograft and primary cancer samples while absent from normal, as expected. Similar results were seen in patient 16 (data not shown).

***MnSOD* Sequencing.** To effect a theoretical oncogenic increase in the level of ROS, the observed mutation of one mtDNA molecule followed by the expansion of that molecule to become the dominant species within the cell would appear to be a less efficient means than would mutation of a nuclear-encoded gene that regulated ROS. For this reason, a sequence analysis of the *MnSOD* gene, coding for an enzyme localized to mitochondria and responsible for neutralizing ROS, was performed. In a survey of 100 xenografted pancreatic tumors, we found LOH of chromosome 6q25.2 in 26% of these tumors. Complete sequencing of the coding sequences of the *MnSOD* gene was performed on the tumors having LOH, and no mutations were discovered.

Discussion

Mutant mtDNA in Cancer: Selection? Mitochondrial DNA exhibits remarkable sequence heterogeneity, as evidenced by the numerous mutations and polymorphisms seen here and collected in the human mitochondrial database. This variability stems from ROS-mediated mutation of mtDNA, resulting in intracellular mtDNA heteroplasmy. As mutations accumulate during life in the mtDNA, oxidative phosphorylation capabilities are sacrificed, and this can contribute to the aging process and disease.

But can preexisting mtDNA heteroplasmy also contribute to cancer? It has been observed that in some heteroplasmic cells, the proportion of mtDNA molecules represented by each segregating mitotype can undergo rapid shifts during cell culture (18). In fact, certain mitochondrial genotypes can quickly and reliably become the dominant mtDNA in a mixed population (9). The mechanism underlying this phenomenon is unknown. In our survey of mutations of the mitochondrial genome in pancreatic cancer, we identified four homoplasmic somatic mutations. In one case, the homoplasmic mutation in the cancer apparently descended from a heteroplasmic population of normal and mutant mtDNAs in the surrounding normal nonneoplastic tissue. According to the classic selection model, heteroplasmic mtDNA mutations may create a subtle functional defect in oxidative phosphorylation that results in slightly increased ROS levels (Ref. 9; Fig. 2*A*). Low levels of ROS are mitogenic to the cell and possibly mutagenic to the nDNA (3). The mutant mtDNA may contribute to a selective advantage during tumorigenesis in cells that have a high copy number of the mutant sequence. It would follow that inherited mutant mtDNA heteroplasmy could be a new basis for genetic cancer susceptibility. The appropriate biological and epidemiological studies would be required to address this possibility. However, there is an alternative hypothesis to explain homoplasmic mtDNA mutations.

Mutant mtDNA in Cancer: Random Drift to Homoplasmy? The sequencing of the mtDNA genome of tumors in this study and in others has confirmed a high mutational load but has not provided incontrovertible evidence of selectable functional effects. Nearly all of the somatic mutations found are missense mutations that are likely to have a subtle, if any, effect upon the integrity of the respiratory electron transport chain. For example, in the case of the mutation we found in tRNA-proline (Table 2), it is very unlikely that this mutation would have any functional alteration. Other studies have even identified somatic mtDNA mutations that are silent, *i.e.*, resulting in usage of an equivalent codon (8–10). It is difficult to imagine that these mutations can confer enough selective advantage to drive a process of intramitochondrial selection followed by intracellular selection, as required to become homoplasmic in the overall cellular mtDNA population (Fig. 2*A*).

A model of random drift to homoplasmy might instead explain the presence of mtDNA mutations seen by our group and others (Fig. 2*B*). During development, mutations to the mitochondrial genome would create intracellular heteroplasmy, a population of many unique mtDNAs, each present at a low frequency. Because of mtDNA "population genetics" alone, at some rate, a number of these mutations will become homoplasmic. Other factors could influence the frequency of

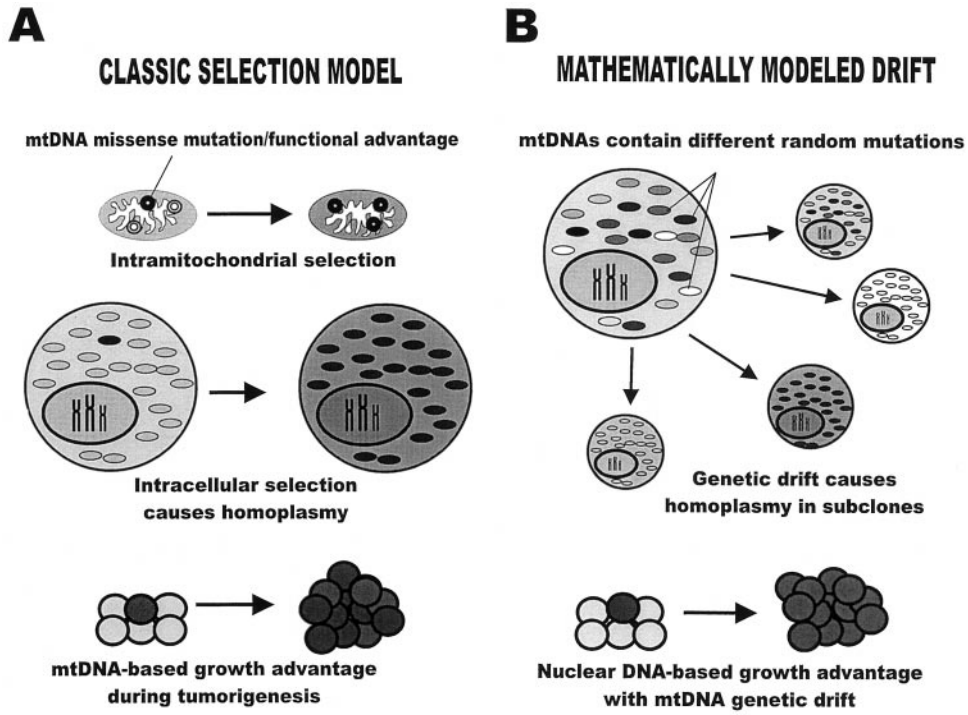

Fig. 2. Schematic comparison of alternative models. *A*, the classic "bi-level" selection model for the acquisition of a homoplasmic mtDNA mutation in a tumor. *Top field*, a particular mutation to a mtDNA genome (*black circle* within light mitochondria at *top left*, wild-type mtDNA indicated by *white circles*) confers an intramitochondrial replicative advantage that allows the mutant mtDNA to become the dominant mtDNA species within a mitochondrion (*top right*). Subsequently, selection acts at the level of the whole mitochondrion whereby mitochondria containing the mutation gain an intracellular replicative advantage. *Middle field*, a normal cell (*left*) has one mitochondrion containing predominantly mutant mtDNA (*dark gray*). After intracellular selection, the cell (*right*) evolves to have homoplasmy (all *dark* mitochondria containing mutant mtDNA). nDNA is represented by a chromosome-containing nucleus. *Bottom field*, cells that are homoplasmic for the mtDNA mutation (*dark* cell on *bottom left*) exhibit a growth advantage that contributes to evolution of tumor (*bottom right*). *B*, genetic drift model for acquisition of a homoplasmic mtDNA mutation in a tumor. *Top field*, a cell (*top left*) contains mitochondria with many unique mtDNAs (different shades of *gray*) because of the high mutation rate of mtDNA. After many generations, the daughter cells generated may retain heteroplasmy of parent cell (cell at *top right*) or through genetic drift may contain one of the mutant mtDNAs in homoplasmy (other cells). *Bottom field*, when one of these cells accumulates nDNA changes sufficient to promote tumorigenesis (*dark* cell on *bottom left*), the tumor (*bottom right*) can exhibit homoplasmic mutant mtDNA.

this event. For example, the proposed "nucleoid model" of mtDNA replication may afford a type of somatic bottleneck, whereby the number of replicating units in each cellular generation is reduced to only the few mtDNA molecules that are part of the replicating nucleoid (19). Additionally, unequal partitioning of daughter mitochondria during cell division could increase the rate at which mutant alleles evolve to homoplasmy. Tumorigenesis, driven in large part through genetic and epigenetic changes affecting the nDNA, involves the clonal expansion of subclones of cells, and with them, their drifted mitotype.

Our statistical modeling of mtDNA segregation in the absence of selection shows that under biologically reasonable conditions, a single mutant mtDNA has the capability to undergo "genetic drift" to become the homoplasmic mtDNA species. This rate can be increased by secondary events, such as a reduction of total replicating units (as in nucleoid model), or reduced number of total mitochondria (effectively the same as an unequal partitioning of daughter mitochondria), as described above. In our simulation, we studied the segregation of a mutation at only one position in the mitochondrial genome. However, given the mutation rate of mitochondrial DNA and the large size of the genome (16.5 kb), the initial somatic cell of the tumorigenic process is likely to contain a considerable number of mutant mtDNA genomes. This suggests that the most likely outcome of random mtDNA evolution in adult-onset tumors might be homoplasmy for one mtDNA mutation.

The likelihood of this premise can be calculated directly from our mathematical modeling. When we began with one mtDNA mutation, we observed that after 2000 generations, 1% of daughter cells contained the mutant mtDNA in homoplasmy. Therefore, inversely, the probability of not generating a homoplasmic mutant cell is 0.99. If the parental cell contained not just 1 but perhaps >50 different mutant mtDNAs, then the probability of not generating a homoplasmic mutant daughter cell would be at most 0.99 raised to the 50th power, *i.e.*, a $<60\%$ chance to avoid homoplasmy for any of the mtDNA mutations. When each cell has such a large likelihood of randomly generating a homoplasmic mutant daughter cell, mathematical modeling hereby supports the hypothesis that most, if not all, of these unique homoplasmic mtDNA mutations seen in our study and others may be explained without invoking selection for mtDNA-based replicative advantage.

More convincing evidence for random drift derives from the observation that normal heteroplasmic cell populations do evolve to homoplasmy *in vivo*. Individuals have been described with constitutional heteroplasmy (Refs. 20 and 21 and Table 2). In these individuals, the proportion of each mitotype exhibits extreme tissue variability (20, 22). For example, Wilson *et al.* (22) examined the relative proportions of two mitotypes in individual hairs, which are derived from very few stem cells, from a proband with inherited heteroplasmy. They found that some hairs had a 1:1 ratio of each mitotype, some hairs had complete homoplasmy of one mitotype, and some hairs had homoplasmy of the other mitotype. Thus, adjacent clones of cells exhibited random drift during development from an initial heteroplasmic state to homoplasmy for either mtDNA allele. Furthermore, in this study we identified at least one unique mtDNA variant present in homoplasmy in nearly all samples sequenced. In conclusion, although selection of subtle mutant mtDNAs may play a role in tumorigenesis, it is important to consider the alternative model: that tumor mtDNAs may exhibit a random drift to homoplasmy (Fig. 2).

mtDNA as Marker of Clonality. Clinical tumor detection methods, designed to identify markers of nuclear genetic lesions, have often failed because of the difficulty of detecting mutant DNA amid the contaminating wild-type DNA present in diagnostic samples. Presumably, the detection of mtDNA lesions would suffer the same consequence because of the same ratio of contaminating normal mtDNA. Fliss *et al.* (8) reported recently the facile detection of mtDNA mutations in diagnostic samples, reliably detecting known mutations of mtDNA against the background of normal mtDNAs present in bodily fluids. There are two potential mechanisms by which this could have occurred. One is that mtDNA in tumor cells might be resistant to degradation, perhaps because of cellular apoptotic defects or other neoplasm-related differences. The alternative would be the possibility that the ratio of mutant:normal mtDNA was altered within

the tumors. Cancers, perhaps being composed of relatively larger cells or because of other physical properties, may simply have more mtDNA than do normal cells. Our Southern blot data support the latter hypothesis by demonstrating the level of mtDNA in two pancreatic cancer samples to be 6–8-fold higher than in normal duodenal cells, when controlled for the level of nDNA. This supports a recent study by Lee *et al.* (23) demonstrating an increase in mtDNA copy number in lung fibroblasts as an early event in response to oxidative stress. Additionally, we confirmed the facile detection of two known mitochondrial mutations by direct sequencing in primary tumors. The malignant cellularity of the two primary carcinomas was low, such that the existence of the known K-*ras* mutations could not be established by direct sequencing (Fig. 1*C*). Strikingly, the known mtDNA mutations were readily detected in the same samples (Fig. 1*C*). In the case of clinical specimens, where the mtDNA mutations are yet unknown, unfortunately, mtDNA is so highly polymorphic that is will be difficult to distinguish the true somatic mutations that designate a tumor from the potential somatic mutations that might occasionally arise during development of a specific tissue. To use mtDNA mutations as a means of detecting clonal populations of cells, it may be necessary to perform sequencing of the entire mitochondrial genome of both a sample containing tumor cells as well as a sample of a tissue-specific normal mtDNA genome of the same patient. This must be done to define the individual average constitutional mitotype amid the wide variability of "normal" mitochondrial genomes seen among the human population. Deviation from the mitotype present in the test sample would indicate the expansion of an alternate mitotype attributable to clonal expansion of a cell containing a high proportion of mutant mtDNA. Thus, despite some caveats, the evolution of homoplasmic mutations in tumors, combined with the relative increase in mtDNA mass quantity, does provide an attractive potential diagnostic marker for further exploration.

References

1. Robin, E. D., and Wong R. Mitochondrial DNA molecules and virtual number of mitochondria per cell in mammalian cells. J. Cell Physiol., *136:* 507–513, 1988.
2. Brown, W. M., George, M., Jr., and Wilson, A. C. Rapid evolution of animal mitochondrial DNA. Proc. Natl. Acad. Sci. USA, *76:* 1967–1971, 1979.
3. Li, Y., Zhou, H., Stansbury, K., and Trush, M. Role of reactive oxygen species in multistage carcinogenesis. *In:* C. Thomas and B. Kalyanaraman (eds.), Oxygen radicals and the disease process, pp. 237–277. Amsterdam: Harwood Academic Publishers, 1997.
4. Kroemer, G., and Reed, J. C. Mitochondrial control of cell death. Nat. Med., *5:* 513–519, 2000.
5. Tallini, G. Oncocytic tumours. Virchows Arch., *433:* 5–12, 1998.
6. Racker, E. Warburg effect revisited. Science (Washington DC), *213:* 1313, 1981.
7. Hauswirth, W. W., and Laipis, P. J. Mitochondrial DNA polymorphism in a maternal lineage of Holstein cows. Proc. Natl. Acad. Sci. USA, *79:* 4686–4690, 1982.
8. Fliss, M. S., Usadel, H., Caballero, O. L., Wu, L., Buta, M. R., Eleff, S. M., Jen, J., and Sidransky, D. Facile detection of mitochondrial DNA mutations in tumors and bodily fluids. Science (Washington DC), *287:* 2017–2019, 2000.
9. Polyak, K., Li, Y., Zhu, H., Lengauer, C., Willson, J. K., Markowitz, S. D., Trush, M. A., Kinzler, K. W., and Vogelstein, B. Somatic mutations of the mitochondrial genome in human colorectal tumours. Nat. Genet., *3:* 291–293, 1998.
10. Yeh, J. J., Lunetta, K. L., van Orsouw, N. J., Moore, F. D., Jr., Mutter, G. L., Vijg, J., Dahia, P. L., and Eng, C. Somatic mitochondrial DNA (mtDNA) mutations in papillary thyroid carcinomas and differential mtDNA sequence variants in cases with thyroid tumors. Oncogene, *19:* 2060–2066, 2000.
11. Caldas, C., Hahn, S. A., da Costa, L. T., Redston, M. S., Schutte, M., Seymour, A. B., Weinstein, C. L., Hruban, R. H., Yeo, C. J., and Kern, S. E. Frequent somatic mutations and homozygous deletions of the *p16* (*MTS1*) gene in pancreatic adenocarcinoma. Nat. Genet., *8:* 27–32, 1994.
12. Hahn, S. A., Hoque, A. T., Schutte, M., da Costa, L. T., Redston, M. S., Caldas, C., Weinstein, C. L., Fischer, A., Yeo, C. J., and Kern, S. E. Allelotype of pancreatic adenocarcinoma using xenograft enrichment. Cancer Res., *55:* 4670–4675, 1995.
13. Anderson, E. A. Sequence and organization of the human mitochondrial genome. Nature (Lond.), *290:* 457–465, 1981.
14. Vogelstein, B., and Kinzler, K. W. Digital PCR. Proc. Natl. Acad. Sci. USA, *96:* 9236–9241, 1999.
15. Cavlier, L., Johannisson, A., and Gyllensten, U. Analysis of mtDNA copy number and composition of single mitochondrial particles using flow cytometry and PCR. Exp. Cell Res., *259:* 79–85, 2000.
16. Cadet, J., Berger, M., Douki, T., and Ravanat, J. L. Oxidative damage to DNA formation, measurement, and biological significance. Rev. Physiol. Biochem. Pharmacol., *131:* 1–87, 1997.
17. Johns, D. R., and Neufeld, M. J. Cytochrome *c* oxidase mutations in Leber hereditary optic neuropathy. Biochem. Biophys. Res. Commun., *196:* 810–815, 1993.
18. Lehtinen, S. K., Hance, N., El Meziane, A., Juhola, M. K., Karhu, R., Spelbrink, J. N., Holt, I. J., and Jacobs, H. T. Genotypic stability, segregation, and selection in heteroplasmic human cell lines containing np 3242 mutant mtDNA. Genetics, *154:* 363–380, 2000.
19. Jacobs, H. T., Lehtinen, S. K., and Spelbrink, J. N. No sex please, we're mitochondria: a hypothesis on the somatic unit of inheritance of mammalian mtDNA. Bioessays, *6:* 564–572, 2000.
20. Bendall, K. E., Macaulay, V. A., and Sykes, B. C. Variable levels of a heteroplasmic point mutation in individual hair roots. Am. J. Hum. Genet., *61:* 1303–1308, 1997.
21. Grzybowski, T. Extremely high levels of human mitochondrial DNA heteroplasmy in single hair roots. Electrophoresis, *3:* 548–553, 2000.
22. Wilson, M. R., Polanskey, D., Replogle, J., DiZinno, J. A., and Budowle, B. A family exhibiting heteroplasmy in the human mitochondrial DNA control region reveals both somatic mosaicism and pronounced segregation of mitotypes. Hum. Genet., *100:* 167–171, 1997.
23. Lee, H. C., Yin, P. H., Lu, C. Y., Chi, C. W., and Wei, Y. H. Increase of mitochondria and mitochondrial DNA in response to oxidative stress in human cells. Biochem. J., *348:* 425–432, 2000.