Somatic mutations of the mitochondrial genome in human colorectal tumours

Kornelia Polyak[1,2,5], Yunbo Li[3], Hong Zhu[3], Christoph Lengauer[2], James K.V. Willson[4], Sanford D. Markowitz[1,4], Michael A. Trush[3], Kenneth W. Kinzler[2] & Bert Vogelstein[1,2]

Alterations of oxidative phosphorylation in tumour cells were originally believed to have a causative role in cancerous growth[1]. More recently, mitochondria have again received attention with regards to neoplasia, largely because of their role in apoptosis and other aspects of tumour biology[2–8] . The mitochondrial genome is particularly susceptible to mutations because of the high level of reactive oxygen species (ROS) generation in this organelle, coupled with a low level of DNA repair[9–12]. However, no detailed analysis of mitochondrial DNA in human tumours has yet been reported. In this study, we analysed the complete mtDNA genome of ten human colorectal cancer cell lines by sequencing and found mutations in seven (70%). The majority of mutations were transitions at purines, consistent with an ROS-related derivation. The mutations were somatic, and those evaluated occurred in the primary tumour from which the cell line was derived. Most of the mutations were homoplasmic, indicating that the mutant genome was dominant at the intracellular and intercellular levels. We showed that mitochondria can rapidly become homogeneous in colorectal cancer cells using cell fusions. These findings provide the first examples of homoplasmic mutations in the mtDNA of tumour cells and have potential implications for the abnormal metabolic and apoptotic processes in cancer.

The human mitochondrial genome is a 16-kb circular, double stranded DNA that encodes 13 polypeptides of the mitochondrial respiratory chain, 22 transfer RNAs and 2 ribosomal RNAs required for protein synthesis. To determine whether mtDNA mutations were present in human colorectal tumours, the entire mitochondrial genome was PCR-amplified from ten human colorectal cancer cell lines in 1–3-kb overlapping fragments, and the PCR products were completely sequenced. The use of large PCR products excluded the possibility that nuclear pseudogenes would complicate this analysis[13]. The sequences obtained were first compared with those recorded in the extensive mitochondrial databank (http://www.gen.emory.edu/mitomap.html). There were 88 sequence variants (4–31 per tumour) that were not recorded in this databank, including 27 variants predicted to alter the amino acid sequence of the encoded protein, 48 variants in protein coding regions predicted to be silent and 13 that affected rRNA or tRNA genes. This database search provided only preliminary evidence for mutations, however, as it could not distinguish somatic mutations from rare germline variants. To make this distinction, we determined which of the variations were present in normal colons from the same patient. This analysis showed that at least seven of the lines contained true somatic mutations. Three of the lines contained a single mutation, and four others contained two or three mutations (Table 1).

Table 1 • Summary of mtDNA mutations

Tumour*	Position	DNA	Mutation	Protein
V478	710	T→C	–	12S rRNA
“	1738	T→C	–	16S rRNA
“	3308	T→C	M1T	ND1
V429	8009	G→A	V142M	COX subunit II
“	14985	G→A	R80H	CYT b
“	15572	T→C	F276L	CYT b
V441	9949	G→A	V248I	COX subunit III
V456	10563	T→C	C32R	ND4L
V425	6264	G→A	G121TER	COX subunit I
“	12418	insA	K28frameshift	ND5
V451	1967	T→C	–	16S rRNA
V410	2299	T→A	–	16S rRNA

*All mutations were homoplasmic, except for those in V451 and V410, which were present in approximately 50% of the mtDNA molecules.

Of the 12 somatic mutations identified, 8 were in protein-coding genes and 4 were in rRNA genes (Table 1). Eleven were nucleotide substitutions and one was a single base pair insertion. Of the eight mutations in protein-encoding genes, one was a nonsense mutation, one was a 1-bp insertion and six were missense mutations (Table 1). All but one of the 11-nt substitutions were T-to-C or G-to-A transitions. This mutational spectrum is fully consistent with the known mutagenic spectra of oxidative damage[14,15].

To determine whether these mutations arose *in vivo* rather than during the process of cell culture, we purified DNA from five of the primary tumours from which the lines were derived (in two cases, no primary tumours were available). In all evaluated cases, the mutation was found in the primary tumour as well as in the cell line (Fig. 1).

Each of the 12 mutations was present in a majority of the mitochondrial DNA molecules, and in 10 of the 12 cases, the mutations were homoplasmic (that is, they were apparently present in every mitochondrial genome; Table 1). This homoplasmy was observed both in primary tumours and cell lines (Fig. 1). These data indicated that a single cell with a mutant mitochondrial genome had acquired a selective growth advantage during tumour evolution, allowing it to become the predominant cell type in the tumour cell population. Furthermore, cells, including the colorectal cancer cell lines studied here (data not shown), each contain hundreds of mitochondria, and each mitochondria contains 1–10 DNA molecules[16]. The homoplasmy therefore indicated that each mutant mitochondrial genome had a replicative advantage in the particular mitochondria in which it occurred, and that this mitochondria had selectively proliferated over other mitochondria in the same cell.

[1]*The Howard Hughes Medical Institute,* [2]*The Johns Hopkins Oncology Center,* [3]*Division of Toxicological Sciences, Department of Environmental Health Sciences, Johns Hopkins University School of Hygiene and Public Health, Baltimore, Maryland 21231, USA.* [4]*Department of Medicine and Ireland Cancer Center, Case Western Reserve University, Cleveland, Ohio 44106, USA.* [5]*Present address: Dana-Farber Cancer Institute, Department of Adult Oncology, Boston, Massachusetts 02115, USA. Correspondence should be addressed to B.V. (e-mail: vogelbe@welchlink.welch.jhu.edu).*

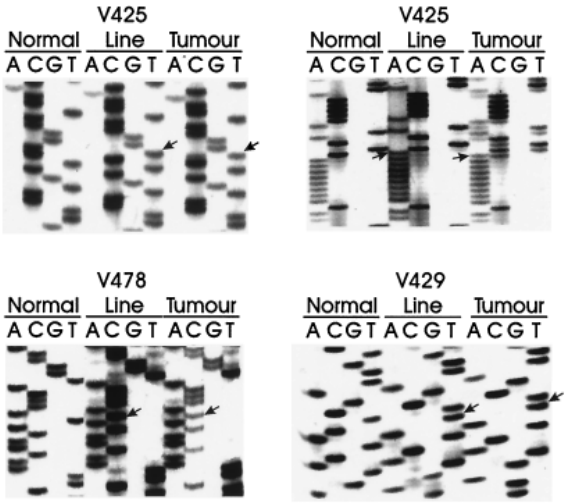

Fig. 1 Examples of mtDNA mutations. The sequence of the mitochondrial genome was determined in normal cells, primary tumours and tumour cell lines from the same patients. Arrows indicate the G→A transition (antisense strand) at codon 121 of the COX subunit I gene (*MTCO1*) in line V425, an A insertion in the $(A)_8$ tract of the ND5 gene (*MTND5*)[32] in line V425, a T→C transition at codon 1 of the ND2 gene (*MTND1*) in line 478 and a G→A transition (antisense strand) at codon 142 of the COX subunit II gene (*MTCO2*) in line V429.

Previous cell fusion experiments have indicated that mitochondria from tumour cells can selectively proliferate when such cells are fused to normal cells[17]. We sought to determine whether a similar mitochondrial dominance could be observed upon fusion between two colorectal cancer cell lines. We first attempted to fuse the lines studied for mitochondrial mutations, but were unsuccessful. We therefore employed more commonly used colorectal cancer cell lines in which intercellular fusions are possible[18]. Geneticin-resistant DLD-1 cells were fused to hygromycin-resistant subclones of each of three different colorectal cancer cell lines (HCT116, HT29 and SW837). The success of fusion was monitored by analysis of nuclear genomic polymorphisms (Fig. 2*a*). Complete sequencing of the mitochondrial genomes revealed 3–7 potential variants in each line; we could not determine which of these were somatic, as normal cells derived from the individuals from whom the cell lines were derived were not available. These variants, however, provided a convenient method to trace the fate of the mtDNA in the fusions. In particular, we used a T→C variant at nt 4,216 that created a restriction endonuclease recognition site for *Nla*III (Fig. 2*b*). The C variant was present in DLD-1 cells, but not in any of the other three lines. DLD-1 mitochondria were 'dominant' over the other mitochondria in each of the fusions (Fig. 2*b*). All three tested clones derived from DLD-1/HCT116 fusions contained mitochondria exclusively of DLD-1 origin. A pool of over 100 stable clones from this fusion also contained only mitochondria from DLD-1 cells. DLD-1 mitochondria were also dominant to those from HT29 and SW837 cells, contributing either all or the majority of mitochondrial genomes in the clones tested (Fig. 2*b*). To determine the time course over which the replicative advantage of DLD-1 mitochondria occurred, we followed pooled clones from DLD-1/HCT116 fusions. Initially, there was a mixture of mitochondrial genomes, with a slight excess of the mitochondria from HCT116 cells. After five days, a skewing towards DLD-1 mitochondria was evident, and a major shift occurred 15–60 days after fusion, by which time only DLD-1 mitochondria remained in the hybrids (Fig. 2*c*). Whether it was strictly the mitochondria, or a combination of nuclear and mitochondrial factors, that was responsible for the selection of DLD-1 mitochondria could not be determined. These experiments clearly documented that tumour mitochondria of one type can have a significant replicative advantage over other types, and are consistent with other experiments documenting the potential for mitochondrial dominance[17].

The mutations we observed generally were transitions affecting G residues, which are the preferred targets for oxidative damage to DNA in general and mtDNA in particular[14,15,19,20] (at least *in vitro*), supporting the idea that the mtDNA mutations resulted from the reactive oxygen species continually generated in mitochondria. We have performed many sequencing analyses of nuclear genes from the same ten lines studied here for mtDNA mutations, and estimate that the prevalence of mutations is at least 10-fold higher in the mitochondrial genome than in the nuclear genome of these cells. Previous experiments have demonstrated large deletions in mtDNA, rather than subtle mutations as observed here, in some tumours[21–25]. No deletions were observed in the lines we studied, despite several attempts to find them using multiple primer pairs in PCR-based strategies. To our knowledge, ours is the first attempt to search for subtle somatic mutations by complete sequencing of the mitochondrial genome.

The mutations we observed were homoplasmic, whereas the deletions observed in tumour cells or normal cells of aging individuals were generally heteroplasmic, present only in a small proportion of the mitochondrial population[21–26]. Our results agree with a previous study[25] in which no somatic mutations in 200 bp of D loop sequence were found. This sequence contains promotor elements for transcription of the mitochondrial genome, whereas the mutations we found were confined to regions encoding mitochondrial proteins or rRNA. What could explain the unexpected homoplasmy of the mutations identified in our study that indicated significant selection at several levels? We propose the following explanation. The mutant somatic mitochondrial genome must be replicated at a higher rate than the wild-type form.

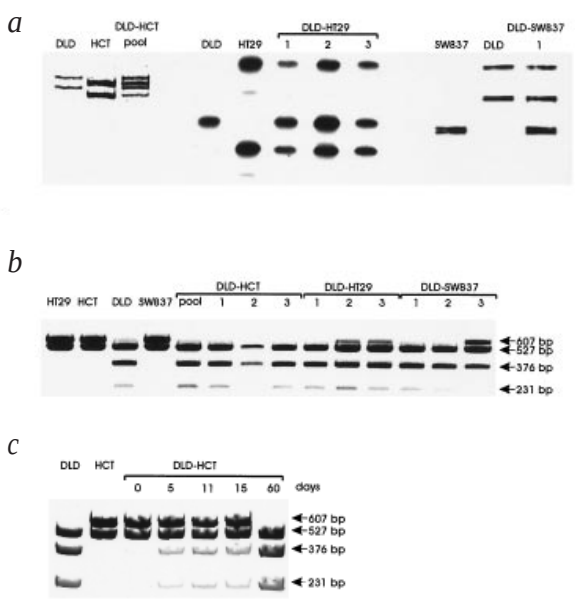

Fig. 2 Somatic cell fusions. ***a***, Confirmation of successful nuclear fusion using nuclear genomic DNA polymorphisms from the indicated lines. ***b***, Analysis of the mtDNA using the T→C variant at nt 4,216 that creates a recognition site for *Nla*III (CATG). The C variant, giving rise to 376- and 231-bp fragments following restriction digest of a 1,140-bp PCR product, is present only in DLD-1 cells. ***c***, Time course over which replicative advantage of DLD-1 mitochondria is evident. Initially, HCT116 cell mitochondria were slightly overrepresented in the fusions, but a shift towards DLD-1 mitochondria was evident within 5 days and this process was complete after 15-60 days. DNA was isolated and the mitochondria were analysed by *Nla*III digestion on the indicated days after cell fusion.

Previous experiments have indicated that replication of mitochondria can be controlled individually, in that signals from aberrantly functioning mitochondria induce their overreplication, perhaps in a compensating effort[27]. Our fusion experiments demonstrate that the process of mitochondrial selection in tumour cells can take place rapidly (Fig. 2*c*). Over the thousands of generations required for tumorigenesis *in vivo*, this process could easily result in the replacement of all mitochondrial genomes in the cell with a mutant form. This cell could then overtake the population through clonal growth, either because the aberrant mitochondria themselves endowed the cell with a selective growth advantage or because that cell sustained a nuclear gene mutation providing such an advantage.

This explanation invokes the idea that the mitochondrial mutations may themselves have a functional effect. We do not believe that most of the changes we observed result in major perturbations of mitochondrial function, as oxygen consumption and the respiratory chain enzymatic activities of several of the lines (Table 1) were largely normal (data not shown). Instead, we propose that these mutations, perhaps in concert with polymorphic variations in mtDNA, result in subtle changes which might generate slightly higher levels of ROS. It has been shown that low levels of ROS are highly mitogenic, whereas high ROS levels are toxic[11]. Regardless of the mechanism for their selection, however, the mutations that we have identified represent a previously unrecognized alteration in tumour cells that could have significant effects on the cellular processes controlled by mitochondria. Their homoplasmy raises questions about the control of mtDNA at the intramitochondrial, intracellular, and cellular population levels. Alterations of tumour mtDNA may also provide clues to their environmental or genetic background, a hypothesis that can be tested in the future using DNA chip technologies[28].

Methods

Cell lines and tumours. Derivation and maintenance of the VACO lines has been described[29]. The DLD-1, HCT116, SW837 and HT29 human colorectal cancer cell lines were obtained from ATCC and maintained in McCoy's medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone) and antibiotics (Gibco).

DNA purification, PCR amplification and sequencing. Cellular DNA from cell lines, primary tumours and normal colonic mucosa was isolated as described[30]. Overlapping fragments (1–3 kb each) of the mitochondrial genome were amplified by PCR using this DNA as template. Manual sequencing of DNA fragments was performed using Thermosequenase (Amersham) and a Genomyx electrophoresis apparatus (Beckman). The sequencing method allowed the detection of any mutation present in more than 25% of the mitochondrial DNA molecules in a given sample. In selected cases, the validity of the sequence data was confirmed using purified mitochondrial DNA as templates. To confirm the mutations in the primary tumours, smaller PCR fragments were generated from the DNA purified from microdissected, paraffin-embedded samples. Each of the 27 sequence variants predicted to result in amino acid changes was evaluated to determine its somatic nature; of these, 8 were found to be somatic and 19 were found in the germ line of the same patient. Of the 13 variants in rRNA or tRNA genes, 9 were evaluated in this way and 4 were found to be somatic. Twenty-five of the forty-eight silent mutations were also evaluated, and none of these were found to be somatic. The primers used for amplification and sequencing are available from the authors upon request.

Cell fusion experiments. Geneticin- or hygromycin-resistant clones were derived through transfection of appropriate plasmid vectors. Approximately 10^6 hygromycin-resistant cells were mixed with an equal number of neomycin-resistant cells and fused by PEG treatment as described[18]. Hybrids were selected in standard growth medium containing geneticin and hygromycin (respectively, 1 mg/ml, 0.25 mg/ml, DLD-1-HCT116 fusion; 1.5 mg/ml, 0.6 mg/ml, DLD-HT29 fusion; 1 mg/ml, 0.25 mg/ml, DLD-SW837 fusion). Successful fusions were verified by nuclear genotyping. Allelotyping was carried out as described[31] using the primer pair wg1g5A/wg1g5B or MapPair primers for *D19S591* and *D16S764* (Research Genetics). Amplified fragments were resolved by electrophoresis in 8% polyacrylamide gels. Reactions using radioactively labelled primers were separated on a 4.5% sequencing gel (Genomyx), whereas reactions using fluorescent labelled primers were analysed on an ABI sequencing system (Perkin-Elmer).

Acknowledgements

This work was supported by the Clayton Fund, the American Cancer Society and NIH grants CA 43460, CA 57345, C A67409, C72160, CA 43703, CA 59366, ES03760 and ES03819.

Received 29 July; accepted 21 September 1998.

1. Warburg, O. On the origin of cancer cells. *Science* **123**, 309–314 (1956).
2. Kroemer, G., Zamzami, N. & Susin, S.A. Mitochondrial control of apoptosis. *Immunol. Today* **18**, 45–51 (1997).
3. Korsmeyer, S.J., Yin, X.M., Oltvai, Z.N., Veis-Novack, D.J. & Linette, G.P. Reactive oxygen species and the regulation of cell death by the Bcl-2 gene family. *Biochim. Biophys. Acta* **1271**, 63–66 (1995).
4. Rudin, C.M. & Thompson, C.B. Apoptosis and disease: regulation and clinical relevance of programmed cell death. *Annu. Rev. Med.* **48**, 267–281 (1997).
5. Wang, H.G. & Reed, J.C. Mechanisms of Bcl-2 protein function. *Histol. Histopathol.* **13**, 521–530 (1998).
6. Cavalli, L.R. & Liang, B.C. Mutagenesis, tumorigenicity, and apoptosis: are the mitochondria involved? *Mutat. Res.* **398**, 19–26 (1998).
7. Chen, L.B. Mitochondrial membrane potential in living cells. *Annu. Rev. Cell Biol.* **4**, 155–181 (1988).
8. Augenlicht, L.H. & Heerdt, B.G. Modulation of gene expression as a biomarker in colon. *J. Cell. Biochem. Suppl.* **16G**, 151–157 (1992).
9. Lightowlers, R.N., Chinnery, P.F., Turnbull, D.M. & Howell, N. Mammalian mitochondrial genetics: heredity, heteroplasmy and disease. *Trends Genet.* **13**, 450–455 (1997).
10. Beal, M. Mitochondria, free radicals, and neurodegeneration. *Curr. Opin. Neurobiol.* **6**, 661–666 (1996).
11. Li, Y., Zhou, H., Stansbury, K. & Trush, M. Role of reactive oxygen species in multistage carcinogenesis. in *Oxygen radicals and the disease process* (eds Thomas, C. & Kalyanaraman, B.) 237–277 (Harwood Academic Publishers, Amsterdam, The Netherlands, 1997).
12. Croteau, D.L. & Bohr, V.A. Repair of oxidative damage to nuclear and mitochondrial DNA in mammalian cells. *J. Biol. Chem.* **272**, 25409–25412 (1997).
13. Parfait, B., Rustin, P., Munnich, A. & Rotig, A. Co-amplification of nuclear pseudogenes and assessment of heteroplasmy of mitochondrial DNA mutations. *Biochem. Biophys. Res. Commun.* **247**, 57–59 (1998).
14. Beckman, K.B. & Ames, B.N. Oxidative decay of DNA. *J. Biol. Chem.* **272**, 19633–19636 (1997).
15. Cadet, J., Berger, M., Douki, T. & Ravanat, J.L. Oxidative damage to DNA: formation, measurement, and biological significance. *Rev. Physiol. Biochem. Pharmacol.* **131**, 1–87 (1997).
16. Wallace, D.C., Brown, M.D., Melov, S., Graham, B. & Lott, M. Mitochondrial biology, degenerative diseases and aging. *Biofactors* **7**, 187–190 (1998).
17. Shay, J. & Ishii, S. Unexpected nonrandom mitochondrial DNA segregation in human cell hybrids. *Anticancer Res.* **10**, 279–284 (1990).
18. Lengauer, C., Kinzler, K.W. & Vogelstein, B. Genetic instability in colorectal cancers. *Nature* **386**, 623–627 (1997).
19. Khrapko, K. *et al.* Mitochondrial mutational spectra in human cells and tissues. *Proc. Natl Acad. Sci. USA* **94**, 13798–13803 (1997).
20. Beckman, K. & Ames, B. Detection and quantification of oxidative adducts of mitochondrial DNA. *Methods Enzymol.* **264**, 442–453 (1996).
21. Welter, C., Kovacs, G., Seitz, G. & Blin, N. Alteration of mitochondrial DNA in human oncocytomas. *Genes Chromosomes Cancer* **1**, 79–82 (1989).
22. Yamamoto, H. *et al.* Significant existence of deleted mitochondrial DNA in cirrhotic liver surrounding hepatic tumor. *Biochem. Biophys. Res. Commun.* **182**, 913–920 (1992).
23. Burgart, L.J., Zheng, J., Shu, Q., Strickler, J.G. & Shibata, D. Somatic mitochondrial mutation in gastric cancer. *Am. J. Pathol.* **147**, 1105–1111 (1995).
24. Tallini, G., Ladanyi, M., Rosai, J. & Jhanwar, S. Analysis of nuclear and mitochondrial DNA alterations in thyroid and renal oncocytic tumour. *Cytogenet. Cell. Genet.* **66**, 253–259 (1994).
25. Heerdt, B.G., Chen, J., Stewart, L.R. & Augenlicht, L.H. Polymorphisms, but lack of mutations or instability, in the promotor region of the mitochondrial genome in human colonic tumors. *Cancer Res.* **54**, 3912–3915 (1994).
26. Wallace, D.C. Mitochondrial DNA sequence variation in human evolution and disease. *Proc. Natl Acad. Sci. USA* **91**, 8739–8746 (1994).
27. Attardi, G., Yoneda, M. & Chomyn, A. Complementation and segregation behavior of disease-causing mitochondrial DNA mutations in cellular model systems. *Biochim. Biophys. Acta* **1271**, 241–248 (1995).
28. Chee, M. *et al.* Accessing genetic information with high-density DNA arrays. *Science* **274**, 610–614 (1996).
29. Parsons, R. *et al.* Microsatellite instability and mutations of the transforming growth factor b type II receptor gene in colorectal cancer. *Cancer Res.* **55**, 5548–5550 (1995).
30. Jen, J. *et al.* Allelic loss of chromosome 18q and prognosis in colorectal cancer. *N. Engl. J. Med.* **331**, 213–221 (1994).
31. Armour, J.A., Neumann, R., Gobert, S. & Jeffreys, A.J. Isolation of human simple repeat loci by hybridization selection. *Hum. Mol. Genet.* **3**, 599–665 (1994).
32. Hofhaus, G. & Attardi, G. Efficient selection and characterization of mutants of a human cell line which are defective in mitochondrial DNA-encoded subunits of respiratory NADH dehydrogenase. *Mol. Cell. Biol.* **15**, 964–974 (1995).