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RESTRICTION ENZYME ANALYSIS OF MITOCHONDRIAL DNA IN COLORECTAL TUMOURS

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Total cellular DNA samples were isolated from 15 colorectal adenocarcinomas, 8 colon adenomas and their adjacent histologically normal colon mucosa. These DNA samples were digested separately with 13 different restriction endonucleases and analysed by Southern blot hybridization using a purified 32P-labelled human mtDNA probe. The fragment patterns from tumour mtDNA were compared to those from corresponding normal mtDNA. No evidence for large deletions, insertions, rearrangements or single base mutations in the detectable regions was detected. This suggests that other mechanisms may be responsible for the changes of colorectal tumour mitochondria.

Mitochondria are the main energy sources in the cell, and occupy 15 to 50 percent of the total cytoplasmic volume. Therefore, they play a very important role in cell growth and proliferation. Significant alterations in structure, distribution and function of mitochondria have been reported in some tumour cells (1-4). In rat hepatoma (5), there is an increase in hexokinase bound to mitochondrial membranes; In colorectal cancer (6), familial adenomatous polyposis (7) and rat hepatoma (8), there are increases in mitochondrial gene expression; In several carcinomas, there is an increased uptake and a prolonged retention of rhodamine 123, a fluorescent mitochondrial-specific dye (9). The reasons for these apparent abnormalities in mitochondria are not understood. Although most of these differences may result from tissue-specific, nuclear coded mitochondrial proteins, some differences may arise from changed mtDNA.

Since development of colorectal neoplasia is associated with widespread deletions in the nuclear genome (10,11), since mitochondrial DNA

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is modified by chemical carcinogens to a greater extent than is the nuclear genome (12-14) and since mitochondria are less efficient in repairing DNA damage and replication errors than nuclei (15), it is necessary to investigate whether some of the mitochondrial abnormalities in tumours are reflected in changes of the mitochondrial genome. In this study, we compared mtDNA from 15 colorectal carcinomas and 8 adenomas to that from corresponding normal mucosa by Southern blot analysis.

MATERIALS AND METHODS

Tissue samples

Fresh tissues were obtained from 15 colorectal carcinomas and 8 adenomas surgically excised from 21 patients at St. Mark's Hospital. For each carcinoma, the tissue samples were obtained from different parts of the tumour: centre, growing edge and surface. For adenomas, the samples were obtained from non-specific regions. All fresh tissue samples were snap frozen in liquid nitrogen after surgical resection, and the diagnosis was confirmed by histopathology. Corresponding normal colon mucosa originated from regions at least 10cm away from the site of tumours.

Preparation of total cellular DNA

Because only small tumour tissues were available, it was difficult to isolate intact mitochondria. Therefore, total cellular DNA was isolated from 20-100mg of the frozen tissues by phenol- chloroform extraction (16).

Preparation of mitochondrial DNA probe

Normal tissue was obtained by sampling fresh liver from a partial hepatectomy specimen in a patient with a solitary isolated hepatic metastatic deposit, and was frozen in liquid nitrogen. Mitochondria were isolated from normal frozen human liver tissue by differential centrifugation as described by Bookelman (17). The mitochondrial DNA was then isolated using a modification of the method of Palva and Palva (18). For a probe, 25ng mitochondrial DNA was linearized with BamHI and labelled in the presence of a-32P deoxycytidine triphosphate with use of the random-primer method (19).

Southern blot hybridization

Approximately 5μg of total cellular DNA was digested with a one to five-fold excess of each restriction endonuclease listed in Table 1. The digestion continued for 16-20 hours at 37°C, then the fragments were separated by electrophoresis through a 0.7-1.5% agarose gel and transferred to the hybridization membrane. The hybridization were performed by 32P-labelled purified human mitochondrial DNA in a hybridization oven at 65°C for 16 hours. The exposure time of autoradiograms was about 2-4 hours to avoid the interference from nuclear genome. The number and size of all fragments produced by each enzyme were deduced using Genetic Computer Group's (GCG) sequence analysis software in the known sequence of human mtDNA.(20)

Table 1. Restriction enzymes, their recognition sites and number of fragments in mitochondrial DNA

Enzyme	Recognition site	Number of fragments
BamHI	G'GATCC	1
EcoRI	G'AATTC	3
HaeII	RGCGC'Y	7
HincII	GTY'RAC	12
HindIII	A'AGCTT	3
HpaI	GTT'AAC	3
HpaII	C'CGG	23
KpnI	GGTAC'C	3
PstI	CTGCA'G	2
PvuII	CAG'CTG	1
SacI	GAGCT'G	2
XbaI	T'CTAGA	5
XhoI	C'TCGAG	1

RESULTS

Autoradiograms of the Southern hybridization are shown in Figures 1 and 2. Each gel in the pictures consists of four lanes which represent DNA from normal mucosa (N), centre of carcinoma (C), growing edge of carcinoma (E) and surface of carcinoma (S). In adenoma, the results consist of two lanes, normal and adenoma, which are not shown here. Identical results were obtained from each of the enzymes tested for 21 patients, so only one gel for each enzyme is shown (Fig. 1). However, some restriction enzymes showed polymorphisms in some patients, the polymorphisms are also shown in the pictures (Fig. 2). The results of XhoI which also produce one band as PvuII pattern I, and the results of EcoRI which gave three bands are not shown here.

In all the cases, there were no observable differences in the fragment patterns or molecular weight of fragments for any of the enzymes between tumour tissues and the corresponding normal tissues, and also no difference between different parts of the carcinoma. In most of the cases (Fig. 1 and Fig. 2I), the bands shown corresponded to the fragments whose sizes were predicted from the GCG computer program. However, in some cases, restriction endonuclease polymorphisms in mtDNA were seen for BamHI, EcoRI, HaeII, HpaII and PvuII nucleases (Fig. 2II) The polymorphism of enzyme EcoRI in one patient was not shown in the figures.

DISCUSSION

To investigate the mitochondrial DNA in colorectal tumours by restriction enzyme analysis, we used thirteen restriction enzymes. They are chosen because they produced a wide size range of restriction

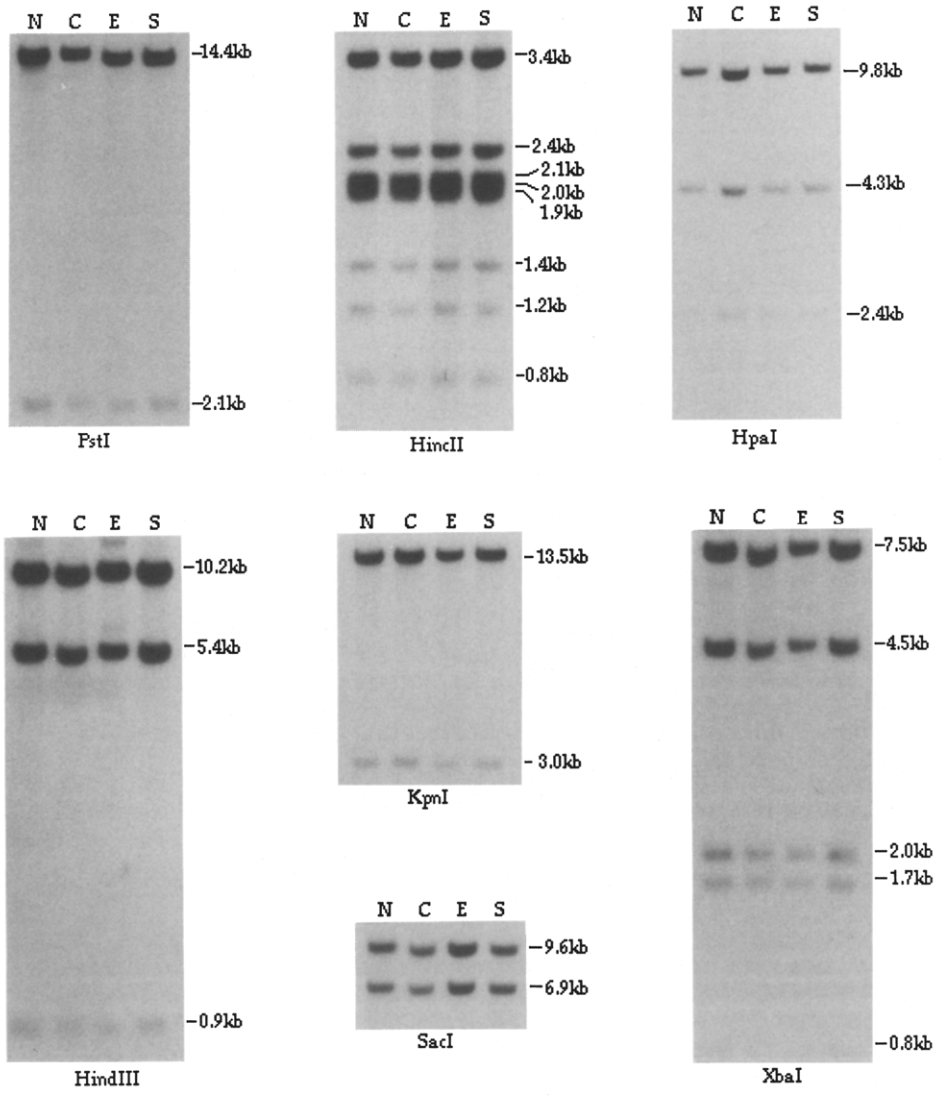

Fig. 1. Southern blot analysis of mtDNA digested with the restriction endonucleases PstI, HincII, HindIII, HpaI, KpnI, SacI and XbaI. 21 patients showed same fragment patterns. No difference was shown between normal and tumour tissues and no difference was shown in different parts of the tumour. N: normal tissue; C, E and S: tissues from the centre, growing edge and surface of the tumour.

fragments, and their recognition sites were distributed relatively randomly around the entire human mtDNA genome. Although single base mutations occurring in the regions between recognition sites would be undetected, the total number of restriction fragments produced by these 13 restriction enzymes would allow for the detection of deletions, insertions or rearrangements in the mtDNA molecules by the appearance or absence of bands in comparison with corresponding normal tissues. As there are variations of mtDNA between individuals, normal mucosa from same patients were chosen as control. We found that no large

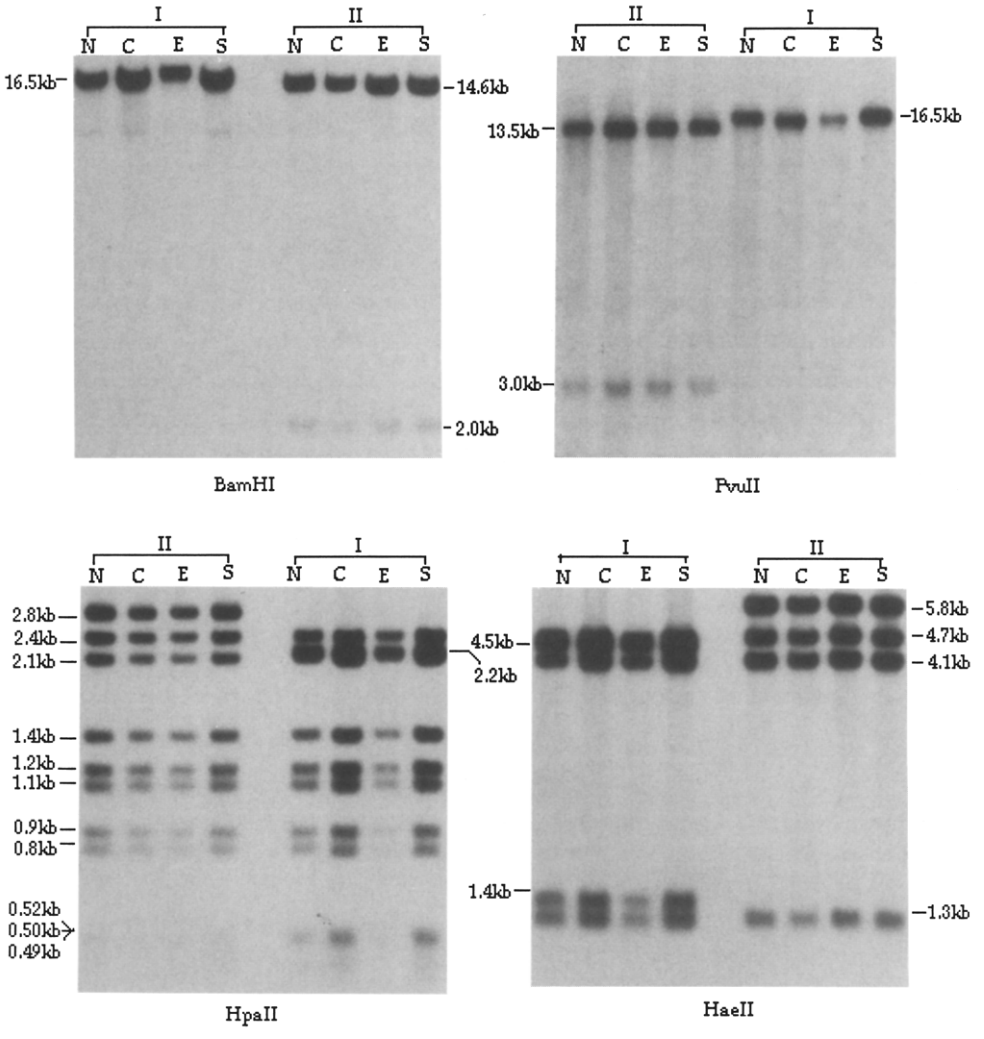

Fig. 2. Southern blot analysis of mtDNA digested with BamHI, HpaII, HaeII and PvuII. For HpaII, 18 patients showed fragment pattern I, 3 patients showed fragment pattern II; for the others, 20 patients showed fragment pattern I, 1 patient showed pattern II. Both pattern I and II showed no difference between normal and tumour tissues.

deletion, insertion and rearrangement in colorectal tumour mitochondrial DNA.

The results here are in accord with the work of Welter and Blin (21) who analysed five human colon adenocarcinomas and found no major structural changes in mtDNA. However, Koike (22) compared mtDNA from a human hepatoma with that from normal placenta by DNA sequencing and found differences in the D-loop region. Recently, Kovacs (23) reported that restriction analysis of the mtDNA from renal oncocytomas revealed a new band. It is possible that those mutations only occurred in some of the mtDNA molecules or some of the tumours.

Mitochondrial DNA restriction polymorphisms have been reported in some populations (24,25), and our results have also shown constitutional mtDNA restriction endonuclease polymorphisms in colorectal tumour patients (Fig. 2). However no particular type of polymorphism is correlated with human colorectal tumours.

Our results suggest that alterations in colorectal tumour mitochondria are not due to deletions or insertions in mitochondtrial DNA As the nuclear genome is partly responsible for the structure and function of mitochondria, further work is needed to determine whether the epithelial cell nuclear DNA deletions and mutations associated with colorectal cancer involve the nuclear sequences which encode the proteins responsible for mitochondrial structure and function.

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