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Advances in Brief

p53 Gene Mutations in Barrett's Epithelium and Esophageal Cancer[1]

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Abstract

Genomic DNA was extracted from archival pathology specimens comprising 10 squamous and 14 adenocarcinomas, including 7 with Barrett's epithelium adjacent to tumor, and corresponding normal esophagus from the resection margin. The polymerase chain reaction was used to amplify selected exons of *p53* which were analyzed for mutations using single-strand conformation polymorphism analysis. Mutations were localized to exon 8 for 1 adenocarcinoma and to exon 5 for 1 squamous tumor and 4 of 7 Barrett's specimens. Sequencing confirmed mutations at codons 273 (CGT→CAT; adenocarcinoma) and 176 (TGC→TTC; squamous) and in Barrett's epithelium at codons 152 (CCG→CTG), 155 (ACC→GCC) and 175 (CGC→CAC). Specimens of Barrett's epithelium from separate sites had identical *p53* mutations suggesting a clonal origin. Cancers arising in mutant epithelium did not have mutations corresponding to those found in the Barrett's specimens suggesting that other events are required for tumorigenesis.

Introduction

Cancer of the esophagus is a particularly virulent gastrointestinal malignancy with poor prognosis. Although the incidence of this cancer in North America is relatively low, it assumes worldwide importance with the wide geographical variation in incidence that is characteristic of this disease (1). Replacement of the normal squamous epithelium of the esophagus with columnar epithelium (Barrett's esophagus) is considered a premalignant condition. Individuals with Barrett's epithelium have a risk of developing esophageal adenocarcinoma that is 30 to 42 times that of the normal population (2, 3). The adenocarcinomas generally originate in the area of Barrett's epithelium. Histological changes ranging from mild dysplasia to carcinoma *in situ* have been detected in Barrett's epithelium. Recently a 10% yearly rate of increase for adenocarcinoma of the esophagus in males was found which exceeds that of any other cancer (4). The *p53* gene has been implicated in a number of human solid tumors, including cancers of the lung and gastrointestinal tract, where it is believed to function as a tumor suppressor gene (5, 6). The aim of these studies was to determine whether point-mutations of the *p53* gene occurred in squamous and adenocarcinomas of the esophagus. Detection of *p53* mutation in esophageal cancers prompted us to study Barrett's epithelium adjacent to esophageal adenocarcinomas to gain insight into the role of this gene in the premalignant stage of this cancer.

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Materials and Methods

Tumor Samples and Isolation of DNA. All specimens were obtained from the Department of Pathology at the University of Texas M. D. Anderson Cancer Center and were reviewed by two staff pathologists (K. R. C., J. Y. R.). Archival specimens comprising formalin-fixed, paraffin-embedded tissue blocks were obtained for 10 esophageal squamous cell cancers and 14 adenocarcinomas, with histologically normal esophagus from the resection margin paired with each tumor. Barrett's epithelium, with varying degrees of dysplasia, was identified in association with 7 of the 14 adenocarcinomas. The remaining 7 adenocarcinomas met strict criteria confirming their esophageal etiology. These included the findings (on endoscopy, on radiology, or at surgery) of at least 75% of the tumor mass in the lower third of the esophagus, invasion of periesophageal tissues, minimal gastric involvement, and the clinical symptom of dysphagia, indicative of esophageal obstruction. All specimens were derived from 24 patients who, between 1988 and 1989, had undergone esophageal resection at our institution.

For each specimen, up to 6 unmounted 5-μm serial sections were prepared. One section was stained with hematoxylin-eosin to facilitate accurate identification of tumor, normal or where available, Barrett's epithelium, which was accurately removed using a scalpel to scrape tissues from each serial slide. This method ensured that only the tissues of interest were removed. For tumors, over 90% of cells removed from each slide appeared histologically malignant, and contamination with adjacent nonmalignant cells was avoided. DNA was extracted as described previously (7). In brief the tissue was dewaxed by 2 washes in xylene and a wash in 70% ethanol. The tissue was suspended in 500 mM Tris-20 mM EDTA-10 mM NaCl, pH 9.0) containing 1% sodium dodecyl sulfate and 500 μg/ml proteinase K. Samples were digested for 24 h at 37°C. Nucleic acid was extracted using 1 volume of phenol-chloroform followed by 1 volume of chloroform. Sodium acetate (0.1 volume of 3 M solution, pH 5.6) was added, and the DNA was precipitated by the addition of 2 volumes of cold absolute ethanol. The DNA was precipitated overnight at −20°C. After centrifugation, the pellet was washed with 70% ethanol and resuspended in 10 mM Tris-1 mM EDTA buffer, pH 7.5.

Up to 83 μg of genomic DNA were extracted from each archival pathology specimen, using the technique described to accurately recover tumor, paired normal esophageal mucosa, and Barrett's epithelium from serial sections of paraffin-embedded tissues. To ensure consistency and reproducibility and to eliminate PCR[4] artifact, all assays were performed on a minimum of three separate occasions, using separate PCRs.

Polymerase Chain Reaction. Amplification of DNA sequences using the polymerase chain reaction was performed as described (8). The *p53* exons previously shown to have a high incidence of mutations were target sequences (9) and included exon 5 (codons 126–187), exon 6 (codons 188–224), exon 7 (codon 225–261), and exon 8 (codons 262–290). Oligodeoxynucleotide amplimers, complimentary to adjacent target sequences, were synthesized on a DNA synthesizer (Applied BioSystem), as follows: exon 5,5′-TACTCCCCTGCCCTCAACAA-3′ and 5′-CATCGCTATCTGAGCAGCGC-3′; exon 6, 5′GTCTGGCCCC-TCCTCAGCAT-3′ and 5′CTCAGGCGGCTCATAGGGCA-3′ exon 7, 5′-TCTGACTGTACCACCATCCA-3′ and 5′-CTGGAGTCTT-CCAGTGTGAT-3′; exon 8, 5′-TGGTAATCTACTGGGACGGA-3′ and 5′-CGGAGATTCTCTTCCTCTGT-3′. The standard PCR reac-

[4] The abbreviations used are: PCR, polymerase chain reaction; SSCP, single-strand conformation polymorphism analysis.

tion was composed of 100 μl reaction volume containing up to 1 μg genomic DNA, 10× Taq polymerase buffer (Stratagene, La Jolla, CA), 1 μM each amplimer, 5 mM each deoxynucleotide triphosphate (dTTP, dGTP, dCTP, dATP) and 1.5 units of Taq DNA polymerase (Stratagene). Mineral oil covered each reaction to prevent evaporation. With the thermal cycler (Ericomp, San Diego, CA), the reaction mixtures underwent cyclical denaturation (92°C, 1 min), annealing (42–51°C depending on the amplimer sequence, 1 min) and elongation (74°C, 1 min). Routinely, 35 amplification cycles were performed, followed by 74°C conditions for 10 min. Agarose gel electrophoresis and Southern analysis were used to confirm amplification of correct target sequences.

Single-Strand Conformation Polymorphism Analysis. SSCP was performed directly by adding 0.5 μl of [α-^{32}P]dCTP (3000 Ci/mmol, 10 mCi/ml; ICN, Irvine, Ca) to the PCR reaction during the last 10 cycles of amplification (10). One-tenth volume of 100 mM EDTA/1.0% sodium dodecyl sulfate was added to each completed reaction. Prior to electrophoresis, 1 μl of the solution was mixed with 1 μl of dye mix (95% formamide-20 mM EDTA-0.05% bromophenol blue-0.05% xylene cyanol), and samples were heated to 80°C prior to loading. Electrophoresis was performed on nondenaturing 12% polyacrylamide (stock 29.2% acrylamide, 0.8% bisacrylamide)-10% glycerol gels, at 30 W for up to 8 h, using a sequencing type apparatus (S2, BRL), with 30- x 40-cm plates and 0.4-mm spacers. After brief fixation with 10% methanol-10% acetic acid, gels were dried (Bio-Rad 583 gel dryer) for 1 h and autoradiography was performed. Each tumor sample was electrophoresed with its corresponding normal tissue to act as an internal control. In addition, each gel included positive and negative controls (tumor with known point-mutation and normal) and a PCR-amplified, labeled complementary DNA to localize the bands of interest.

Subcloning and Sequencing. Sequence analysis was used to confirm and localize point-mutations detected by SSCP. Eluted PCR-amplified DNA (300 ng) was ligated overnight at room temperature with 100 ng (1 μl) of *Sma*1 (BMB)-digested Bluescript vector (Stratagene) using 5 units of DNA ligase (BMB). Ligated DNA was used to transform DH5α competent cells which were plated onto ITPG/X-gal (BMB) ampicillin agar plates. Following overnight incubation, selected individual white colonies were expanded, and plasmid DNA extracted by Miniprep. Plasmid DNA was purified using the Prep-A-Gene DNA purification kit (Bio-Rad, Richmond, CA) prior to sequencing.

Sequencing double-stranded DNA templates, using the chain termination method, were performed using a Sequence Version 2.0 kit (USB), following the manufacturer's protocol. The SK primer of the Bluescript vector (5′-TCTAGAACTAGTGGATCC-3′) was used to sequence PCR-amplified DNA from single clones. A minimum of 5 individual clones were sequenced for each specimen.

Results

***p53* Mutations in Esophageal Cancer.** All esophageal tumors were screened for point-mutations in exons 5–8 of the *p53* gene, using SSCP. An electrophoretic mobility shift between tumor and its paired normal tissue (internal control) was characteristic of a point-mutation. Such differences were detected in exon 5 for one squamous cell tumor (Fig. 1*A*), and in exon 8 for one adenocarcinoma (Fig. 2*A*).

Sequencing confirmed a G to T transversion at codon 176 (exon 5) for the squamous tumor (Fig. 1*B*) and a G to A transition at codon 273 (exon 8) for the adenocarcinoma (Fig. 2*B*).

***p53* Mutations in Barrett's Epithelium.** Barrett's epithelium was present adjacent to esophageal adenocarcinomas in 7 specimens, and exons 5–8 of *p53* were screened for mutations by SSCP for each specimen. Mobility shifts of Barrett's epithelium (relative to control normal esophagus and corresponding tumor) were found in 4 specimens and were all localized to exon 5 (Fig. 3*A*). Histologically, each of these 4 specimens comprised

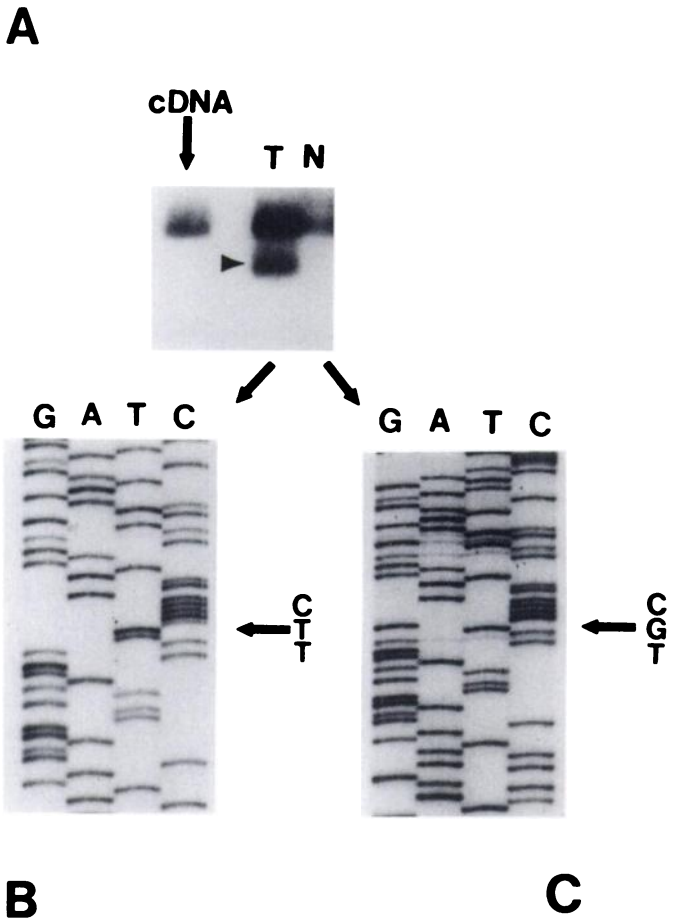

Fig. 1. Detection of *p53* point-mutation in an esophageal squamous cell tumor (patient 1). *A*, mobility shift between tumor (*T*) and its paired normal tissue (*N*) on SSCP analysis. A PCR-amplified complementary DNA marker was used to identify the amplified bands of interest. *B*, sequencing confirmed a point-mutation (GT) at codon 176 for this tumor. *C*, wild-type *p53* sequence found in paired normal esophageal epithelium.

Barrett's epithelium only, with minimal low-grade, or no dysplasia.

Sequencing confirmed G to A point-mutations at codon 175 for two specimens, an A to G point-mutation at codon 155, and a C to T point-mutation at codon 152. These data are summarized in Table 1.

To investigate whether *p53* mutations were consistent throughout the Barrett's epithelium, SSCP analysis was used to screen three different regions sampled from two Barrett's specimens (Table 1, patients 4 and 5) that had been shown to have point-mutations. The electrophoretic mobility shift of SSCP was identical for each specimen, indicating that such mutations were consistent throughout the Barrett's epithelium sampled (Fig. 3*B*). Identical point-mutations were found in PCR DNA for each of the samples from the same Barrett's specimen. A minimum of 5 separate clones sequenced from each sample showed only the mutant *p53* gene without detectable wild-type sequences. Similarly, no mobility shifts were detected by SSCP performed on Barrett's epithelium sampled from two different regions of a mutation-negative specimen (Fig. 2*A*, patient 3). Selected normal esophageal and tumor samples that did not exhibit mobility shifts on SSCP (patients 4, 5, and 11) were sequenced also. No corresponding mutations were detected, and the wild-type sequence was found in each case.

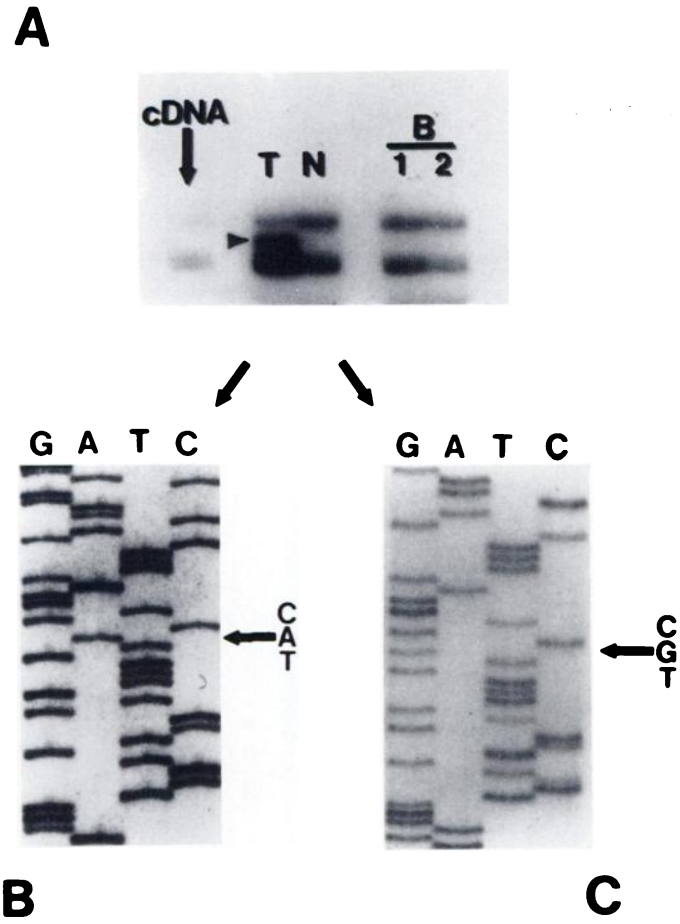

Fig. 2. *p53* point-mutation in an esophageal adenocarcinoma (patient 3). *A*, mobility shift detected by SSCP analysis for the tumor (*T*). Paired normal tissue (*N*) and Barrett's epithelium sampled from two different regions (*B1, B2*) adjacent to tumor showed no mobility shift. *B*, sequencing confirming a point-mutation (GA) at codon 273 for this tumor. *C*, normal *p53* sequence.

Discussion

The molecular genetic events underlying the development of human esophageal cancer are unknown. Experimental studies have implicated H-*ras* in rat esophageal tumorigenesis (11), and expression of the *ras* oncogene p21 protein has been reported in over 80% of human esophageal squamous cell carcinomas studied by immunohistochemistry (12). However, no activating point-mutations of the *ras* gene were reported in studies of human esophageal (squamous) tumors from high-incidence regions of South Africa (13), France (14), or China (15). We also did not detect point-mutations of *ras* in this patient population (data not shown).

One study recently reported *p53* gene mutations in human esophageal cancer cells in 5 of 14 surgically resected esophageal squamous tumors from France (16). Mutations were localized to exons 5, 6, 7, and 8, in keeping with the observation that mutations tend to occur in conserved regions of the gene. Only two tumors were found to have point-mutations in our study (1 of 10 squamous; 1 of 14 adenocarcinomas), localized by SSCP to exons 5 and 8. Mutations at codon 273, the site of mutation for the esophageal adenocarcinoma, have also been described in colon, brain, breast, and lung tumors, whereas mutations at codon 176, the site of mutation of the esophageal squamous tumor, have been reported only in non-small lung cancers (9, 17–20). However, this latter codon is immediately adjacent to a region (codon 175) where point-mutations have been reported for brain, breast, and colorectal tumors. Sequence analysis of at least 5 individual clones from each sample consistently demonstrated the mutated form of the *p53* gene, suggesting loss of the wild-type sequences. The techniques used to study these esophageal specimens permitted accurate recovery of tumor, normal or Barrett's epithelium from archival pathology specimens. That only the tissues of interest were recovered using these techniques ensured that samples were not mixed with adjacent tissues, which could potentially diminish a mutated signal. The recovery of DNA from formalin-fixed, paraffin-embedded blocks increases the applicability of such techniques to the study of other tumors, utilizing archival pathology material (7). PCR has been demonstrated by these studies to be a rapid, reliable method of amplifying target DNA sequences of interest (8). Concern regarding the possibility of PCR-mediated base misincorporation were allayed by repeating such assays on several occasions and by using different initial PCR reactions. We used PCR-SSCP to screen selected exons of *p53* (up to 200 base pairs) for point-mutations and found it to be a satisfactory screening technique for such a diverse gene (10). Results were confirmed by sequencing exons in which mutations were suspected, and we encountered no false-positive results. Further evaluation of SSCP is required, however, to ensure that all mutations are detected, avoiding false-negative results.

The concept of the premalignant nature of the columnar epithelium-lined esophagus originated from the observation that some esophageal adenocarcinomas arose from ectopic gastric mucosa (21). Two studies specifically addressed the risk of subsequent development of adenocarcinoma from Barrett's epithelium and estimated the risk to be 30 to 42 times that of the general population (2, 3). Controversary exists as to the correct management of Barrett's epithelium in light of this increased risk of cancer development, uncertainty as to the potential for high-grade dysplasia to progress to carcinoma, and whether these changes can be controlled by medical or surgical treatment.

Barrett's epithelium was found adjacent to 7 of the 14 esophageal adenocarcinomas studied. Point-mutations of the *p53* gene were detected in 4 of these 7 specimens, each with minimal or no dysplasia, and were localized to exon 5 at codons 153, 155, and 175 (Table 1). *p53* point-mutations have been reported at codon 175 in brain and colorectal cancers, but point-mutations at the adjacent codons 152 and 155 (Barrett's specimens) have not been reported in other human cancers. The grouping of reported mutations in exon 5 would tend to suggest a diffuse "hot region" in this exon. To our knowledge, *p53* mutations have not been previously identified in premalignant lesions. Germ line *p53* mutations have recently been reported in a familial cancer syndrome (22). The prevalence of G to A and C to T transitions suggests that the mutagens involved are different from those implicated in lung cancer (19, 20). This is in agreement with the lack of association between adenocarcinoma and tobacco use.

Point-mutations were found in Barrett's epithelium sampled from different regions of the same specimen, and thus the mutations did not appear to have site specificity. The finding of identical mutations in separate regions of the same Barrett's specimen suggests that the lesion has a clonal origin. Interestingly, mutations were not detected in the corresponding adenocarcinomas associated with these mutation-positive Barrett's specimens and by contrast were not detected in Barrett's epi-

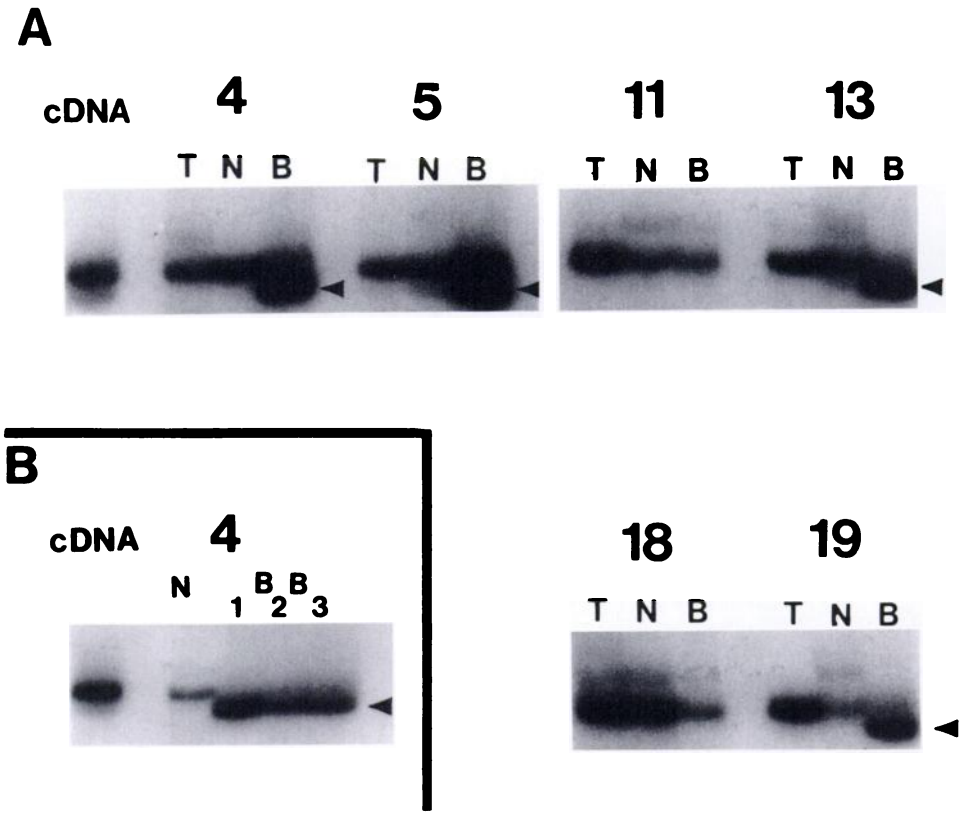

Fig. 3. SSCP analysis of exon 5 of the *p53* gene. *A*, mobility shifts of Barrett's epithelium specimens (*B*) were detected for 4 patients (4, 5, 13, and 19), relative to normal esophageal epithelium (*N*), tumor (*T*), and a PCR-amplified complementary DNA marker. Mobility shifts were not detected in this exon for patients 3 (not shown), 11, and 18. *Inset B*, further SSCP analysis of Barrett's epithelium (*B1, B2, B3*) from patient 4. Mobility shifts were detected relative to the complementary DNA marker and corresponding normal esophageal tissue (*N*). Consistency of results is illustrated by similar mobility shifts for Barrett's samples amplified by two separate PCRs (*B1 versus B2* and *B3*) and by analysis of Barrett's epithelium sampled from two separate regions of the specimen (*B2* and *B3*).

Table 1 *p53 mutations in human esophageal cancer and Barrett's epithelium*

Patient	Histology	SSCP	Exon	Codon Base	Amino acid
1	Squamous	+	5	176 TGC→TTC	cys→phe
3	Adenocarcinoma	+	8	273 CGT→CAT	arg→his
	Barrett's[a]	−			
4	Adenocarcinoma				
	Barrett's[a]	+	5	152 CCG→CTG	pro→leu
5	Adenocarcinoma	−			
	Barrett's[a]	+	5	175 CGC→CAC	arg→his
13	Adenocarcinoma	−			
	Barrett's[a]	+	5	175 CGC→CAC	arg→his
19	Adenocarcinoma	−			
	Barrett's	+	5	155 ACC→GCC	thr→ala

[a] Two different regions screened. SSCP was used to detect point-mutations, which were characterized by electrophoretic mobility shifts (+). −, no shift.

thelium (two regions) adjacent to the only adenocarcinoma in which a point-mutation was detected. The frequency of *p53* mutations in Barrett's epithelium may reflect the suspectibility of proliferating esophageal epithelium to mutational events. The acquisition of a *p53* mutation may confer a growth advantage to the proliferating cell. The proliferative characteristics of premalignant esophageal epithelium was addressed by a recent study, which reported a progressive increase in cell proliferation from normal to severely dysplastic esophageal epithelium (23). It is also conceivable that the malignant cells may influence the growth rate of surrounding dysplastic cells, thereby predisposing it to mutational events. Mutations of the *p53* gene were detected in 2 of 24 esophageal cancers and may contribute to their development. The development of tumors that are negative for *p53* mutations suggests that other oncogenes may be involved in esophageal tumorigenesis. The high frequency of point-mutations detected in Barrett's epithelium of patients with esophageal tumor implies that *p53* gene mutations may be useful predictors of patents at high risk for developing cancer.

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