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Evaluation of *p53* mutations in premalignant esophageal lesions and esophageal adenocarcinoma using laser capture microdissection

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_p53_ mutations have been implicated in the development of esophageal malignancies. The purpose of this study was to assess more accurately the incidence and types of _p53_ mutations in Barrett's esophagus (BE) with and without dysplasia and in esophageal adenocarcinoma, using pure preparations of epithelial cells obtained by laser capture microdissection (LCM). Assays were performed on paraffin-embedded tissue samples of normal antrum and premalignant and malignant esophageal samples from 57 patients, including 16 controls, 10 with BE metaplasia alone, 20 with BE-associated dysplasia, and 11 with BE-associated adenocarcinoma. All tissues were processed for LCM. DNA was extracted from isolated cells, and polymerase chain reaction (PCR) was performed using oligonucleutide primers for exons 5–8 of _p53_. PCR products were processed for DNA sequencing. _p53_ sequence abnormalities were identified in 2/16 cases of normal antrum and regenerative/chemical gastritis, 1/10 cases of BE, 1/20 cases of BE with dysplasia, and 2/11 cases of adenocarcinomas. The abnormalities occurred in exons 7 and 8 in the form of point mutations. Our results, using LCM, show that _p53_ gene mutations are relatively rare in esophageal preneoplastic and neoplastic conditions. Only point mutations were detected, but no deletions/insertions were identified.
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Barrett's esophagus (BE) develops through a metaplastic process in which an abnormal columnar epithelium replaces the normal stratified squamous epithelium of the distal esophagus.[1–3] Furthermore, this lesion predisposes to the development of dysplasia and adenocarcinoma of the esophagus.

The prediction of which patients with Barrett's metaplasia will progress to malignancy is difficult. A better genetic characterization of this condition may help clinicians to determine the risk of cancer development and to elaborate an adequate preventive strategy as well as better treatment and follow-up plans.

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Mutations of the tumor-suppressor gene *p53* have been implicated in the pathogenesis of esophageal carcinomas. The purpose of this study was to assess accurately the incidence and types of *p53* mutations in BE with and without dysplasia and in esophageal adenocarcinoma, using pure preparations of epithelial cells obtained by laser capture microdissection (LCM).

Materials and methods

Tissue Samples

A total of 57 formalin-fixed paraffin-embedded samples were retrieved from the surgical pathology files of Beth Israel Deaconess Medical Center, Boston, MA, USA, with patients' ages ranging from 33 to 84 years. Specimens were obtained from patients with normal antrum (10), regenerative/chemical gastritis (six), BE without dysplasia (10), BE with dysplasia (20), and adenocarcinoma of esophagus (11).

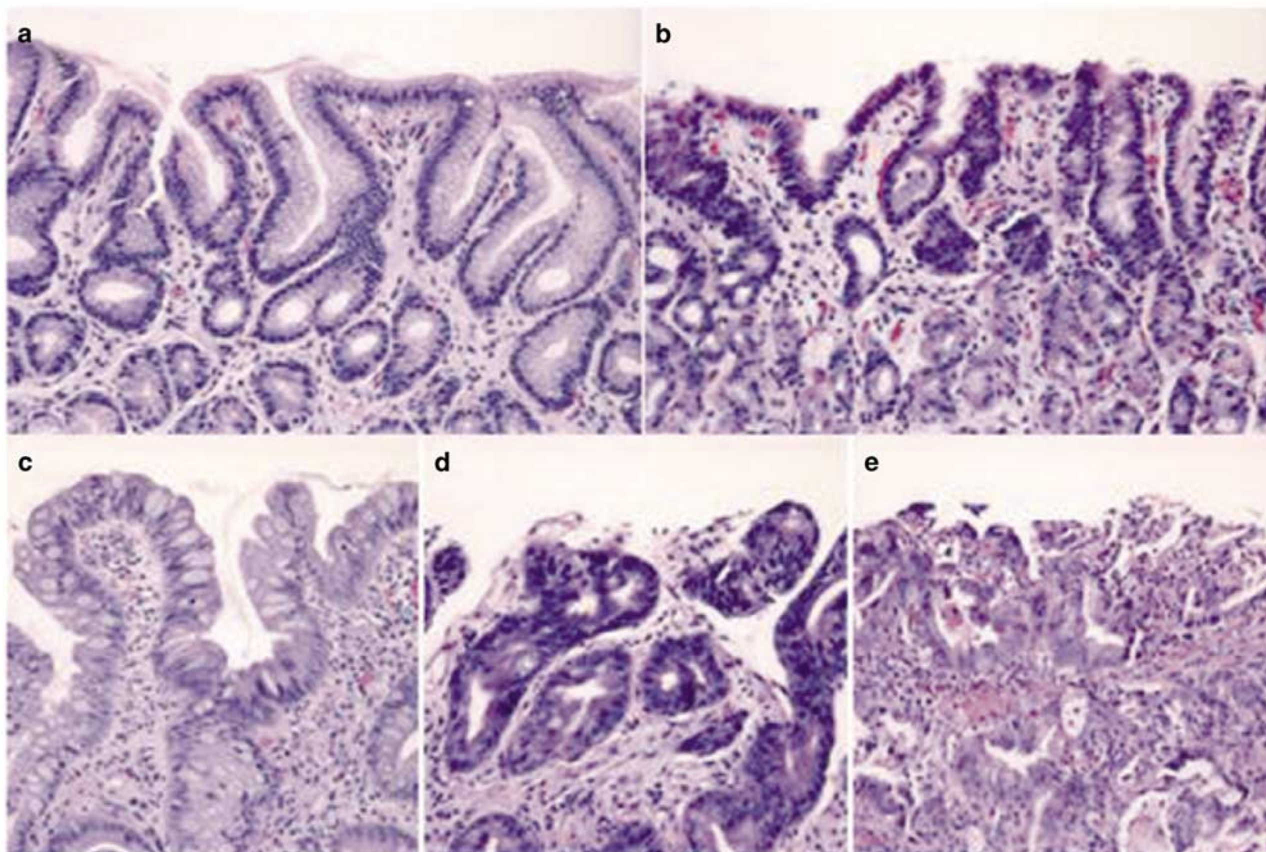

Figure 1 Hematoxylin and eosin-stained tissue sections of: (**a**) normal antrum; (**b**) regenerative/chemical gastritis; (**c**) Barrett's esophagus; (**d**) dysplasia; (**e**) adenocarcinoma of esophagus ($\times$10).

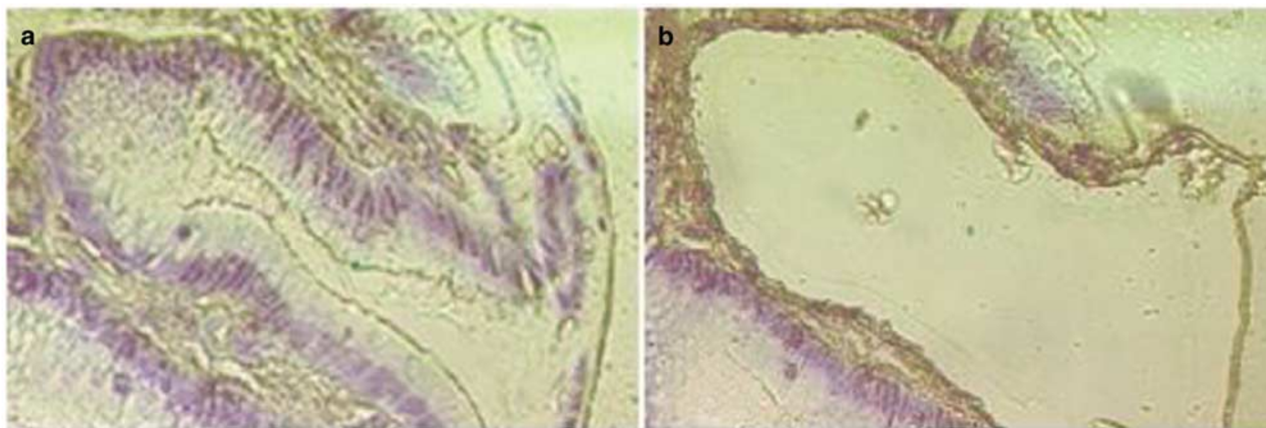

Figure 2 Toluidine-blue-stained sections of normal antrum before (**a**) and after (**b**) LCM.

Histopathologically, regenerative/chemical gastritis is associated with regeneration and hyperplasia of the surface and foveolar mucous cells as well as the absence of neutrophils in the lamina propria.[4] Dysplastic specimens are classified as 'low-grade dysplasia' (with decreased mucus secretion, crowding of slender columnar cells with pseudostratified nuclei, and occasional mitosis; pleomorphism is absent or mild; the glands retain the normal contour but may be enlarged), and 'high-grade dysplasia' (where the criteria include moderate pleomorphism, plump cells, marked reduction of mucus secretion, and frequent mitosis; the glands show budding, branching, crowding, and intraluminal infolding). Adenocarcinomas are graded as well and poorly differentiated.

Samples were used for LCM, polymerase chain reaction (PCR), and DNA sequencing (Figure 1).

LCM

All 57 formalin-fixed paraffin-embedded samples were recut on glass slides coated with LPC-membrane (PEN Foil 1.35 μm, P.A.L.M. Microlaser Technologies, Bernried, Germany) and deparaffinized. Slides were then stained with toluidine blue and used for LCM (Figure 2). Pure cell populations of normal antrum, regenerative gastric glands, and Barrett metaplastic, dysplastic, and adenocarcinomatous glands were obtained from our samples by LCM (P.A.L.M., Microlaser Technologies).

DNA Extraction

DNA was extracted from isolated cells using the PURGENE DNA Isolation Kit protocol and resuspended in 50 μl of ddH_2O (Gentra System, Minneapolis, MN, USA).

PCR Amplification

Exons 5–8 of the *p53* gene (the sites of most somatic *p53* mutations) were amplified by PCR using amplification primers from published sequences[5] as follows: exon 5: 5′-ttcctcttcctgcagtactc-3′ and 5′-cagctgctcaccatcgct-3′, exon 6: 5′-cactgattgctcttaggt-3′ and 5′-agttgcaaaccagacctc-3′, exon 7: 5′-gttggctctgactgtaccaccat-3′ and 5′-gctcctgacctggagtct-3′, and exon 8: 5′-cctatcctgagtagtggt-3′ and 5′-tcctgc-ttgcttacctcgct-3′. The size of PCR DNA fragments were: 190, 126, 96, and 165 bp, respectively.

An amount of 10 μl of DNA was subjected to 40 cycles of PCR in a volume of 50 μl containing 50 ng of each oligonucleotide primer, 0.5 U *Taq* DNA polymerase, 8 μl of dNTP at 1.5 mM, 24.5 μl dH_2O, and 5 μl of 10 × PCR buffer containing 1.5 mM $MgCl_2$ (Applied Biosystems, Foster City, CA, USA). PCR conditions were 94°C for 1 min, 53°C for 2 min, and 72°C for 1 min.

DNA fragments from PCR were then purified using the QIAquick PCR Purification Kit Protocol (QIAGEN, Valencia, CA, USA), and electrophoresed on 2% agarose gel.

Table 1 *p53* mutation during neoplastic progression of BE in 57 cases

	N	*Point mutation*	*Insertion/ deletion*
Normal antrum & regenerative/ chemical gastritis	16	2	0
Barrett's esophagus	10	1	0
Dysplasia	20	1	0
Adenocarcinoma	11	2	0

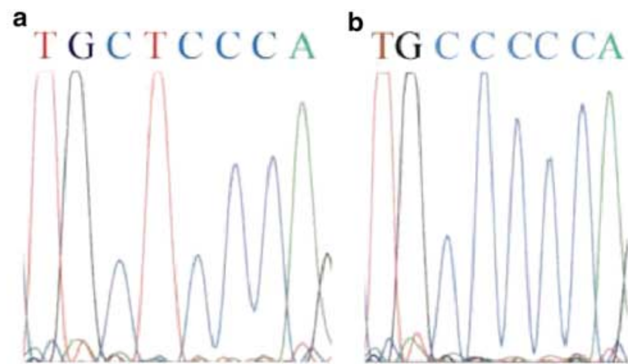

Figure 3 DNA sequence of exon 8 of *p53* gene: point mutation in codon 300 in a case of well-differentiated adenocarcinoma (case #54) with C→T substitution (**a**), compared to normal sequence (**b**).

DNA Sequence Analysis

Amplified DNA fragments were sequenced using an ABI prism 373 Sequencer (Applied Biosystems) in both directions.

Statistical Analysis

A two-way contingency table with Fisher's exact correction (StatView for Windows, SAS Institute Inc., version 5.0.1, Cary, NC, USA) was used to compare the incidence of *p53* mutations among the lesions studied.

Results

Point mutations of *p53* were identified in six of the 57 cases studied (Table 1). They were detected in two of the 16 (12.5%) normal/regenerative stomach; in one of the 10 (10%) BE; in one of the 20 (5%) low-grade and high-grade dysplasia; and in two of the 11 (18%) esophageal adenocarcinoma. There was no

Table 2 *p53* mutation during neoplastic progression of BE

Case	*Histology*	*Exon*	*Codon*	*Mutation*	*Amino acid*
7	Normal antrum	8	275	tgt→cgt	C→R
14	Normal antrum	8	294	gag→gaa	E→E
19	Barrett's esophagus	7	249	agg→aag	R→K
31	Low- and high-grade dysplasia	7	249	agg→aag	R→K
51	Poorly differentiated adenocarcinoma	8	277	tgt→tat	C→Y
54	Well-differentiated adenocarcinoma	8	300	ccc→ctc	P→L

C = cysteine; E = glutamic acid; K = lysine; L = leucine; P = proline; R = arginine; Y = tyrosine.

significant difference in the incidence of point mutations between normal/regenerative antrum and BE without dysplasia ($P > 0.05$), between BE without dysplasia and BE with dysplasia ($P > 0.05$), and between BE with dysplasia and adenocarcinoma ($P > 0.05$). These results suggest that *p53* gene mutations are relatively rare in esophageal preneoplastic and neoplastic conditions. In all these cases, *p53* gene mutations were in the form of point mutations involving exons 7 and 8 (Table 2, Figure 3). Deletions/insertions were not identified.

Of the two mutations present in normal antrum, one of them was a 'silent' mutation and did not result in a change of amino-acid sequence (Table 2, case 14). All other mutations resulted in a change of amino-acid sequence, one in normal antrum, one in BE, one in dysplastic Barrett's epithelium, and two in invasive adenocarcinomas (Table 2).

Discussion

Carcinogenesis in metaplastic esophageal columnar cells begins with genetic alterations that activate proto-oncogenes, disable tumor suppressor *genes*, or involve both mechanisms.[6] The evolution of genetic changes leading from BE to adenocarcinoma is incompletely understood. However, several studies have shown that this neoplastic progression includes alterations in the tumor suppressor *genes p53* and *p16* and nonrandom losses of heterozygosity (LOH).[7–10] In addition to these changes, tetraploid populations are observed in more than 90% of adenocarcinomas.[8]

A relatively low frequency of *p16* (retinoblastoma) gene mutation has been reported in patients with BE and esophageal adenocarcinoma.[11] Cytogenetic alterations have also been studied by comparative genomic hybridization (CGH). Frequent losses on the Y chromosome of 4q, 5q, 9p, 18q, 7q, and 14q and gains on 8q, 20q, 2p, 7p, 10q, 6p, 15q, and 17p were detected in metaplastic BE and esophageal adenocarcinoma, and a correlation between an increase in abnormalities and progression toward dysplasia and adenocarcinoma has been noted.[12] DNA content, measured by flow cytometry, has been also the subject of numerous studies, reporting that aneuploidy and increased G2M/tetraploid populations may increase along with increasing dysplasia, but the results have been conflicting.[13,14]

The prevalence of *p53* gene mutation in esophageal carcinoma reported in the literature ranges from 42 to 67%,[6,15–17] with the exception of two reports in which a low prevalence (8%) *(18)* and a higher prevalence (84%),[19] respectively, were observed. The prevalence of protein accumulation is more variable, ranging from 34 to 87%.[15–17] The discordance between immunohistochemistry (high frequency of positively staining cases) and molecular techniques (relatively low number of *p53* gene mutations) reported in the literature ranged between 24 and 45% of cases[16,20–22] of esophageal carcinoma. This discordance between *p53* phenotype/genotype expression raises a problem in the interpretation of *p53* accumulation demonstrated by immunohistochemistry as an indirect sign of *p53* mutations.

In our study, using LCM, we provided a more precise assessment of the incidence and types of *p53* mutations in BE with and without dysplasia and in esophageal adenocarcinoma. Indeed, selection of areas of interest and precise microdissection of those focal areas permitted us to obtain pure epithelial cell preparations. Our results, contrary to the majority of publications, did not show a significant increase in the incidence of *p53* gene mutation in premalignant and malignant esophageal lesions in the exons that we screened. This is probably due to the purity of our cell population obtained by selection of specific cells by LCM, and direct sequencing of PCR products in both directions, rather than using indirect techniques.[7,12,15,16,18,23–29] Therefore, the role of *p53* mutation as a prognostic factor in progression of metaplastic BE toward esophageal adenocarcinoma is not certain. Since the molecular basis of *p53* function and mutations is not fully understood, a better evaluation of the biological properties of different *p53* mutations is needed in order to interpret the results. Also, the hypothesis of mutations arising at different stages in the evolution of the BE is of concern, since we found no statistically significant increase in mutations compared to controls. However, our findings are based on a relatively limited number of cases, and a large multicenter study is needed to better evaluate the role of *p53* alterations in progression of BE and as a prognostic factor.

In conclusion, the results of our study show that mutations of *p53* using exons 5–8 are relatively rare in esophageal preneoplastic and neoplastic conditions and therefore are of limited use as a marker to study disease progression.

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