

p16^{INK4a} Lesions Are Common, Early Abnormalities that Undergo Clonal Expansion in Barrett's Metaplastic Epithelium¹

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ABSTRACT

Barrett's esophagus (BE) is the only known precursor to esophageal adenocarcinoma, a cancer of which the incidence has been increasing at an alarming rate in Western countries. *p16*^{INK4a} lesions occur frequently in esophageal adenocarcinomas but their role in neoplastic progression is not well understood. We detected 9p21 loss of heterozygosity, *p16* CpG island methylation, and *p16* mutations in biopsies from 57%, 61%, and 15%, respectively, of 107 patients with BE. In contrast, no mutations were found in *p14*^{ARF} or *p15*, and methylation was found in only 4% and 13%, respectively. >85% of Barrett's segments had clones with one (*p16*+/-) or two (*p16*-/-) *p16* lesions. Both *p16*+/- and *p16*-/- clones underwent extensive expansion involving up to 17 cm of esophageal mucosa. The prevalence of established biomarkers in BE, such as 17p (*p53*) loss of heterozygosity, aneuploidy, and/or increased 4N (tetraploid) populations, increased from 0% to 20% to 44% in patients whose biopsies were *p16*+/, *p16*+/-, and *p16*-/-, respectively ($P < 0.001$). Barrett's segment lengths also increased with change in *p16* status with a median of 1.5, 6.0, and 8.0 cm for patients with *p16*+/, *p16*+/-, and *p16*-/- biopsies, respectively ($P < 0.001$). We conclude that most Barrett's metaplasia contains genetic and/or epigenetic *p16* lesions and has the ability to undergo clonal expansion, creating a field in which other abnormalities can arise that can lead to esophageal adenocarcinoma.

INTRODUCTION

BE⁴ is the only known precursor to esophageal adenocarcinoma, a cancer of which the incidence has been increasing rapidly in the United States and other regions of the Western world (1). BE is a condition in which metaplastic columnar epithelium replaces the normal stratified squamous epithelium of the esophagus as a complication of chronic gastroesophageal reflux (2–4). This premalignant metaplasia has been reported to be hyperproliferative, clonal, and involving variable lengths of the distal esophagus, but the basis for these biological behaviors is not understood.

The frequency of *p16*^{INK4A} lesions is second only to *p53* abnormalities in human cancer in general and esophageal adenocarcinoma in particular (5–7). *p16* is inactivated by a two-hit mechanism that can involve CpG island methylation, 9p21 LOH, mutation, or homozygous deletion (8–10). We and others (11–14) have reported previously that *p16* can be inactivated by CpG island methylation, 9p21 LOH, or mutation in esophageal adenocarcinomas, but homozygous deletions have rarely been reported in this condition. *p16* abnormalities have also been detected in premalignant tissues (11, 15–22) including the epithelium surrounding a variety of malignancies such as esophageal

adenocarcinoma, suggesting that they arise before the development of cancer. However, the stage at which these lesions arise and their role in neoplastic progression has not been determined previously.

We assessed *p16* lesions in “mapped” endoscopic biopsy specimens from 107 patients with BE who had not developed cancer. We found *p16* CpG island methylation, 9p21 LOH, and/or *p16* mutations in biopsies from >85% of patients at all of the histological grades of progression in BE. *p16* genotype was strongly correlated with Barrett's segment length, and both *p16*-/- and *p16*+/- clonal cell populations had the ability to expand over extensive regions of Barrett's epithelium, creating a field of abnormal epithelial cells.

MATERIALS AND METHODS

Patients and Endoscopic Biopsies. We evaluated flow-sorted samples from multiple biopsies of Barrett's epithelium in 107 patients who had BE but did not have cancer. Of the 107 patients, 83 were male (78%) and 24 were female (22%). The mean age of the patients was 64.0 years (range, 36.3–83.1). Biopsies were acquired at 2-cm intervals throughout the Barrett's segment by using the endoscopic mapping protocols described previously (23–25). Normal gastric tissue served as a constitutive control for each patient. Patients were counseled regarding the risks and benefits of endoscopic surveillance and informed of potential alternatives, including surgery for high-grade dysplasia. The BE Study was approved by the Human Subjects Division of the University of Washington in 1983 and renewed annually thereafter with reciprocity from the Fred Hutchinson Cancer Research Center since 1993.

Histology. Endoscopic biopsies were processed and interpreted for grade of dysplasia, and patients were classified according to the maximum histological grade of dysplasia in any biopsy, as described previously (24, 25). The categories of indefinite for dysplasia and low-grade dysplasia were combined because observer variation studies have shown that they cannot be reproducibly distinguished (24). Of the 107 patients, 41 were negative for dysplasia, 45 had changes in the indefinite for dysplasia/low-grade dysplasia range, and 21 had high-grade dysplasia.

Flow Cytometric Sorting and DNA Extraction. Barrett's epithelial cell populations were purified from biopsies acquired at 2-cm intervals of the Barrett's segment by using Ki67/DNA content flow sorting of diploid G1, 4N, and aneuploid cell populations on a Coulter Elite ESP cell sorter, as described previously (26–28).

DNA Methylation Analysis. Extracted genomic DNA from flow-purified samples obtained at 2-cm intervals in the Barrett's segment was evaluated for methylation of the *p16*, *p15*, and *p14*^{ARF} CpG islands by using a modification of the methods for bisulfite treatment and MSP described previously (13, 29). Briefly, bisulfite-treated DNA was purified with reverse phase extraction by using Empore SDB-XC disc cartridges (3M, St. Paul, MN). Each purified bisulfite-treated DNA sample was divided into two PCR reactions: 1) PEP, as described previously (for *p15* and *p14*^{ARF} MSP) and 2) *p16*-specific PCR (for *p16* MSP and sequencing). Primer sequences for *p16*-specific PCR were 5'-GTA GGT GGG GAG GAG TTT AG-3' (sense) and 5'-TCC AAT TCC CCT ACA AAC TTC-3' (antisense). The *p16*-specific PCR reaction used 1.75 mM MgCl₂, 200 μM dNTPs, 10 pmol of each primer, GeneAmp PCR buffer (Applied Biosystems, Foster City, CA), and 2.5 units of AmpliTaq Gold enzyme (Applied Biosystems). Cycle conditions were 95°C for 10 min followed by 45 cycles of 95°C for 30 s, 61°C for 30 s, and 72°C for 1.5 min, with a final extension at 72°C for 10 min. Primer sequences and reaction conditions for *p15* and *p14*^{ARF} MSP were as described previously (30, 31). Methylation-specific

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⁴ The abbreviations used are: BE, Barrett's esophagus; LOH, loss of heterozygosity; MSP, methylation-specific PCR; PEP, primer-extension preamplification; dNTP, deoxynucleotide triphosphate; HGD, high-grade dysplasia.

Table 1 Prevalence of p16 lesions at each histologic grade

A. p16 lesion					
	n	9pLOH	p16 mutation	p16 methylation	Any p16 alteration
Metaplasia	41	20 (49) ^b	3 (7)	27 (66)	36 (88)
IND/LGD	45	28 (62)	10 (22)	21 (47)	39 (87)
HGD	21	13 (62)	3 (14)	17 (81)	18 (86)
All grades	107	61 (57)	16 (15)	65 (61)	93 (87)

B. p16 genotype				
	n	+/+	+/-	-/-
Metaplasia	41	5 (12)	24 (59)	12 (29)
IND/LGD	45	6 (13)	23 (51)	16 (36)
HGD	21	3 (14)	7 (33)	11 (53)
All grades	107	14 (13)	54 (51)	39 (36)

^a IND/LGD, indefinite for dysplasia/low-grade dysplasia; HGD, high-grade dysplasia.

^b Numbers in parentheses are %.

primer sequences designed for the p16 CpG island spanned seven CpG cytosines (bold and italicized). The primer sequences of p16 for the unmethylated reaction were 5'-TAG AGT AGG TAG TGG GTG GT-3' (sense) and 5'-CTC CAA CCA TAA CTA TTC AAT ACA-3' (antisense), and the primer sequences of p16 for the methylated reaction were 5'-TAG AGT AGG TAG CGG GCG GC-3' (sense) and 5'-TCC GAC CGT AAC TAT TCG ATA CG 3' (antisense). A 1:60 dilution of the p16-specific PCR reaction was used for each p16 MSP reaction. These primers were designed to target the regions of the p16 promoter which initially undergo methylation in model systems (29). We compared results obtained by using these new primers with those obtained by using primers designed by Herman *et al.* (30). Of 96 samples, 82 (85%) showed identical results; of those that differed, there were 4 examples where the Herman primers detected methylation whereas the new primers did not, and 10 examples where the converse was true (data not shown). Neither the Herman nor the new primer set has generated false positives in extensive testing. We concluded that the results obtained by using the new primers are equivalent to those used by Herman *et al.* (30). The annealing temperature for both methylated and unmethylated reactions was 59.5°C. All of the MSP reactions used 1.5 mM MgCl₂, 200 μM dNTPs, 10 pmol of each primer, GeneAmp PCR buffer, and 2.5 units of AmpliTaq Gold enzyme. Human genomic DNA treated *in vitro* with Sss I methyltransferase (New England Biolabs, Beverly, MA) was used as the methylated control for all three CpG islands.

Methylation of each individual CpG cytosine in the p16 CpG island was also assessed by directly sequencing PCR products of bisulfite-treated DNA in a subset of cases. Nested PCR reactions were performed by using each p16-specific PCR product. The primer sequences for the 5' end of the p16 CpG island were 5'-CAG GAA ACA GCT ATG ACC GTA GGT GGG GAG GAG TTT AGT T-3' and 5'-TGT AAA ACG ACG GCC AGT TCT AAT AAC CAA CCA ACC CCT CC-3', which were tailed with the M13 reverse and forward primer sequences, respectively (italicized). Primer sequences for the 3' end of the p16 CpG island were 5'-CAG GAA ACA GCT ATG ACC GAG GGG TTG GTT GGT TAT TAG-3' and 5'-TGT AAA ACG ACG GCC AGT TCC AAT TCC CCT ACA AAC TTC-3', which were also tailed with the M13 sequences (italicized). Annealing temperatures were 64°C and 60°C, respectively. Both nested PCR reactions used 1.5 mM MgCl₂, 200 μM dNTPs, 10 pmol of each primer, GeneAmp PCR buffer, and 1.25 units of AmpliTaq Gold DNA polymerase. Nested PCR products were purified by using Microcon 100 (Amicon) and then sequenced with M13 forward primers by using BigDye Terminator cycle sequencing (Applied Biosystems) on an Applied Biosystems Incorporated 377 DNA sequencer.

DNA Sequencing. Genomic DNA extracted from ~1000 cells was whole-genome-amplified by using PEP, as described previously (27). Exon 2 of the p16 gene was amplified by using PCR from an aliquot of the PEP product with the following primer sequences: 5'-GGA AAT TGG AAA CTG GAA GC-3' and 5'-TCT GAG CTT TGG AAG CTC T-3'. Reaction conditions were 2.0 mM MgCl₂, 200 μM dNTPs, 14 pmol of each primer, 5% DMSO, GeneAmp PCR buffer (Applied Biosystems), 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems), and an annealing temperature of 66°C. p16 exon 2 PCR products were sequenced by using BigDye Terminator cycle sequencing (Applied Biosystems) on a ABI 377 DNA sequencer. All of the mutations

were confirmed with at least two independent PCR reactions. Wild-type sequences were confirmed for all of the patients by using constitutive samples.

Microsatellite LOH Analysis. PEP DNA from flow-purified samples obtained at 2-cm intervals in the Barrett's segment was evaluated for 9p21 and 17p LOH by using polymorphic microsatellite markers, as described previously (27, 32). The LOH data used in this study are a subset of those described previously in Galipeau *et al.* (32).

Statistical Methods. The relationship between Barrett's segment length and increasing p16 lesion genotype ($p16+/+ \rightarrow p16+/- \rightarrow p16-/-$) was evaluated by using a nonparametric test for trend across ordered groups (33). Differences in segment length between the unordered groups defined by single p16 alteration type were evaluated with the Kruskal-Wallis test (34). A test for the linear-by-linear association of increasing p16 lesion genotype with increasing histological abnormality (Table 1B) used a log-linear modeling approach (35). The relationship between the p16 genotype and the prevalence of at least one of 17p LOH, aneuploidy, or tetraploidy was evaluated by testing the contribution of an ordered covariate for p16 genotype to a logistic regression model of marker prevalence.

RESULTS

p16 lesions were detected in the Barrett's segments of 93 of 107 patients (87%; Fig. 1 and 2; Table 1A). p16 CpG island methylation,

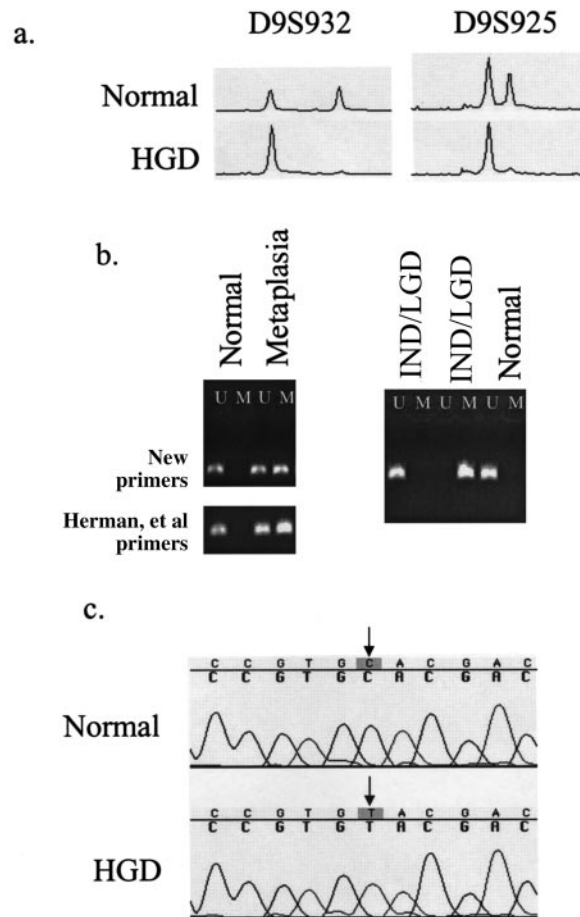


Fig. 1. p16 alterations in patients with BE. *a*, chromatograms demonstrating LOH at loci flanking the p16 locus in a biopsy from a patient diagnosed with HGD. The same pattern of loss was found throughout the 11-cm Barrett's segment. *b*, determination of methylation status by using MSP of bisulfite-treated DNA. The figure on the left demonstrates the similar signals obtained by using the new primers for assaying for p16 promoter methylation and by using the standard primers described by Herman *et al.* (30). The figure on the right demonstrates representative methylation data obtained from two biopsy samples and a gastric normal from a patient diagnosed as IND/LGD. One level of this patient's 3-cm Barrett's segment displayed promoter hypermethylation (right), whereas the other was unmethylated (left). *c*, mutation (H83Y) of the p16 gene in a patient with HGD. This mutation was found throughout the 11-cm Barrett's segment.

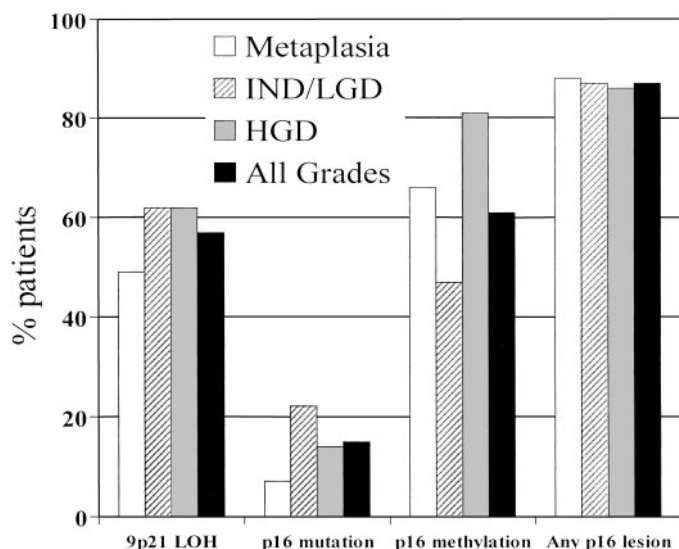


Fig. 2. Prevalence of *p16* lesions in patients with BE relative to histological grade. Metaplasia refers to patients with a maximum diagnosis of metaplasia negative for dysplasia. IND/LGD refers to patients with a maximum diagnosis of indefinite for dysplasia/low-grade dysplasia. HGD refers to patients with a maximum diagnosis of high-grade dysplasia. All of the grades combine all 107 patients.

p16 mutation, and 9p21 LOH were detected in 65 (61%), 16 (15%), and 61 (57%) patients, respectively. Samples with no *p16* alterations were designated *p16*+/+ (wild-type), those with only one alteration *p16*+/- (hemizygous), and those with two alterations *p16*-/- (nullizygous). Patients were categorized according to the maximum number of alterations in any biopsy (*p16*-/- > *p16*+/- > *p16*+/+). In the 107 patients, the maximum *p16* genotype was *p16*+/+ in 14 (13%), *p16*+/- in 54 (51%), and *p16*-/- in 39 (36%; Table 1B). Of 152 distinct cell populations in the Barrett's segments of the 107 patients, 30 (20%) were wild-type, 79 (52%) were hemizygous, and 43 (28%) were nullizygous.

The prevalence of *p16* abnormalities did not change with advancing grade of dysplasia. We found *p16* lesions in 86–88% of patients at all of the histological grades (Fig. 2). The prevalence of each *p16* lesion (*p16* CpG island methylation, *p16* mutation, and 9p21 LOH) was also similar at each grade of dysplasia, indicating that *p16* abnormalities develop at an early stage of neoplastic progression in BE. There was no evidence of an association between *p16* genotype and grade of dysplasia ($P = 0.26$; Table 1).

We used "mapped" endoscopic biopsies to investigate the distribution of *p16*+/- and *p16*-/- clones in the Barrett's segment. Clones with *p16* lesions were found at all or all but one levels of the Barrett's segment in 88 of the patients (82%), creating a field within the esophageal mucosa. We found clonal expansion of cell populations with lesions in one (*p16*+/-) or both *p16* alleles (*p16*-/-; Fig. 3, a and b). For example, one patient had a clonal *p16*+/- cell population with the same *p16* mutation occupying a Barrett's segment of 7 cm, and a second patient had a clonal cell population with the same 9p LOH pattern that expanded to 7 cm (Fig. 3a). Similarly, we found another patient with a cell population with only *p16* methylation that had expanded to 9 cm of esophageal mucosa (Fig. 3a). We also found expanded *p16*-/- clonal cell populations. In one patient, a clone with the same 9p LOH pattern and the same *p16* mutation had expanded to occupy an 11-cm Barrett's segment (Fig. 3b). In another, a clone with the same mutation and methylation occupied 8 cm of a 9-cm Barrett's segment and, in a third patient, a clone with the same LOH pattern and methylation expanded to occupy an entire 7-cm segment (Fig. 3b).

Nullizygous *p16*-/- progeny clones appeared to evolve from

hemizygous progenitors having only a single *p16* lesion (*p16*+/-). Fifteen patients had evaluable *p16*+/- and *p16*-/- cell populations in their Barrett's segment. In 14 of the 15 cases (93%), the *p16* lesions in the *p16*-/- population included the lesion found in the *p16*+/- population, suggesting that the hemizygous population was the progenitor of the nullizygous progeny. For example, we found a patient who had a clonal *p16*+/- population with 9p21 LOH adjacent to a *p16*-/- population with the same 9p LOH pattern and a mutation of the remaining *p16* allele (Fig. 3c). In another patient, a clonal *p16*+/- population with 9p21 LOH was adjacent to a *p16*-/- population with the same 9p LOH pattern and methylation of the remaining *p16* allele. In a third patient, a *p16*+/- population with *p16* methylation was adjacent to a *p16*-/- population with methylation and 9p21 LOH. Thus, Barrett's epithelium can be a mosaic of progenitor and progeny clones with one and two *p16* lesions, respectively.

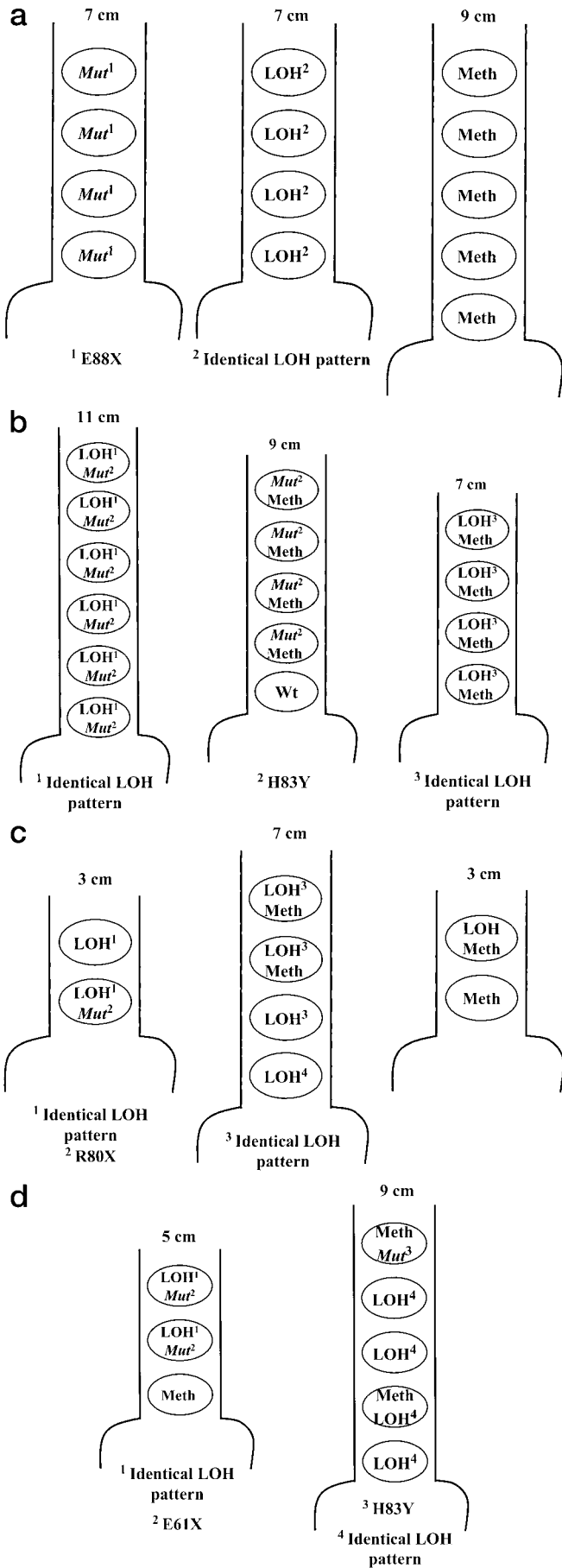
In a minority of cases ($n = 5$), we found mosaics in which two clones appeared to have arisen independently. For example, we found a patient with a methylated *p16* cell population next to a cell population with 9p21 LOH and a *p16* mutation that together occupied a 5-cm segment, as well as a patient with a cell population with 9p21 LOH adjacent to a cell population with *p16* mutation and methylation that together occupied a 9-cm Barrett's segment (Fig. 3d).

We also investigated the relationship between *p16* genotype and Barrett's segment length. We found that the median Barrett's segment lengths for *p16*+/+, *p16*+/-, and *p16*-/- genotypes increased progressively from 1.5 to 6.0 to 8.0 cm, respectively ($P < 0.001$). Because it is possible that different alterations in *p16* may have different effects on segment length, we then examined the correlation between segment length and alteration type in those patients having only one type of alteration present in their Barrett's segment. The median segment lengths for patients with no alterations ($n = 14$), methylation alone ($n = 19$), LOH alone ($n = 17$), or mutation alone ($n = 5$) were 1.5, 5, 7, and 7 cm, respectively. Whereas a significant difference in median segment length was detected between these four groups ($P = 0.05$), this was attributable primarily to the difference of the single alteration groups relative to the *p16* wild-type group. If restricted to differences between the three single alteration groups, there was no evidence of an association between single alteration type and segment length ($P = 0.23$).

Finally, data for 17p (*p53*) LOH, aneuploidy, and increased 4N (tetraploid) fractions, all of which have been shown to predict progression to esophageal adenocarcinoma in patients with BE (25, 36, 37), were available in the same "mapped" endoscopic biopsies of the 107 patients in this study. A total of 28 patients had 17p (*p53*) LOH, aneuploidy, and/or increased 4N, including 20 with LOH, 12 with aneuploidy, and 13 with increased 4N. These abnormalities were only found in patients who had *p16* lesions. None of 14 patients whose biopsies were *p16* wild-type had 17p LOH, aneuploidy, or increased 4N compared with 11 of 54 patients (20%) whose biopsies were *p16* hemizygous and 17 of 39 patients (44%) whose biopsies were *p16* nullizygous ($P < 0.001$).

DISCUSSION

The metaplastic epithelium of BE is premalignant, hyperproliferative, and spreads to involve variable lengths of the distal esophagus, but the biological basis for these abnormalities has not been understood. We found somatic lesions of the *p16* tumor suppressor gene in biopsies of more than 85% of patients with BE. Cell populations with hemizygous lesions (*p16*+/-) were more common (52%) than nullizygous cell populations (28%), yet both underwent extensive clonal expansion, occupying as much as 17 cm of esophageal mucosa. Nullizygous progeny clones (*p16*-/-) appeared to have evolved from



hemizygous progenitor clones ($p16^{+/-}$), suggesting that neoplastic progression in BE may be associated with two waves of clonal expansion as the first and second $p16$ alleles are progressively inactivated. Finally, we found that $p16$ genotype was highly correlated with both median Barrett's segment length and the prevalence of 17p ($p53$) LOH, tetraploidy, or aneuploidy.

The most striking phenotype of $p16$ lesions in BE *in vivo* was clonal expansion. Our finding that 82% of patients had clones with $p16$ lesions at all or all but one levels of the Barrett's segment is consistent with a previous study (21) that demonstrated extensive areas of $p16$ methylation in six esophagectomy specimens. Our results also extend earlier studies of $p16$ in BE as well as other premalignant epithelia of the head and neck, bladder, and lung (13, 16–18, 32, 38). In several studies, cell populations with $p16$ lesions (including 9p LOH or $p16$ methylation) have been shown to undergo extensive expansion within the epithelial linings of these organs (21, 39, 40). Thus, early acquisition of $p16$ lesions with subsequent clonal expansion may be a common step in the development of many human epithelial tumors, in addition to BE.

Although there are at least three candidate tumor suppressor genes on 9p21, including $p16$, $p15$, and $p14^{ARF}$, our results suggest that $p16$ is the target for inactivation in BE. Whereas 9p LOH would be expected to eliminate all three of these genes, $p16$ was found to be specifically inactivated by methylation, mutation, or both in 68% of the cases examined (data not shown). None of the $p16$ mutations we found affected $p14^{ARF}$, and we and others (11, 41) have previously sequenced $p15$ without detecting mutations. In this present study, we also determined the prevalence of methylation of the $p15$ and $p14^{ARF}$ CpG islands in the Barrett's segment of 53 patients by MSP. Only 7 (13%) had $p15$ methylation and only 2 (4%) had $p14^{ARF}$ methylation, compared with 37 (70%) having $p16$ methylation (data not shown). Our data are consistent with recent reports (22, 42) that the $p14^{ARF}$ CpG island is rarely methylated in BE and esophageal cancers. Thus, in contrast to gliomas, leukemias, and a subset of primary colorectal carcinomas (31, 43), we found little evidence for the involvement of $p15$ and $p14^{ARF}$ in neoplastic progression in BE.

BE develops as an adaptive response to chronic injury and denudation of the esophageal mucosa, and our results demonstrate that most Barrett's epithelium are a clonal proliferation rather than polyclonal regeneration of acid reflux-damaged mucosa. These data suggest that clones with $p16$ lesions spread to replace epithelium damaged as a result of gastroesophageal reflux, but the basis for selection of populations with $p16$ lesions is unclear. $p16$ mediates an early stage of cellular senescence *in vitro* (44–48), and it is possible that abrogation of this stage of senescence is required for cell populations to expand and replace damaged esophageal epithelium in BE. This interpretation is consistent with our observations that inactivation of a single $p16$ allele is sufficient for clonal expansion and is supported by a previous study (29, 48) in primary human cell culture that found partial down-regulation of $p16$ expression was sufficient for life span extension. Another cyclin-dependent kinase inhibitor, $p27^{Kip1}$, has also been shown to be haploinsufficient for tumor suppression (49), but, in contrast to $p27$, inactivation of both copies of $p16$ is common in primary human tumors (9, 10, 13). Indeed, all of the 7 patients who developed cancer during follow-up in our study were $p16^{-/-}$ before cancer was detected (data not shown); however, our finding that $p16$

Fig. 3. $p16$ lesions in premalignant Barrett's epithelium from "mapped" endoscopic biopsies in patients without cancer. Each diagram represents the esophagus of an individual patient. The length of the segment of Barrett's epithelium in each patient is indicated above the diagram. Each ○ represents a single "mapped" endoscopic biopsy that was analyzed for $p16$ methylation (Meth), $p16$ mutation (Mut), and 9p21 LOH (LOH). Wt, wild-type $p16$.

hemizygous progenitors gave rise to *p16* nullizygous progeny may indicate that both *p16*^{+/-} and *p16*^{-/-} clones are selected during neoplastic progression.

Other investigators (50, 51) have shown that the severity of gastroesophageal reflux correlates with length of the Barrett's segment, but the genetic factors that contribute to segment length have remained unknown. We found that Barrett's segment length was significantly correlated with *p16* genotype, increasing from a median length of 1.5 to 6.0 to 8.0 cm in wild type, hemizygous, and nullizygous segments, respectively. These results, combined with our observations that the prevalence of 17p (*p53*) LOH, aneuploidy, and increased 4N are correlated with *p16* genotype, provide a somatic genetic mechanism for observations that increasing Barrett's segment lengths are associated with a trend toward increased risk of neoplastic progression (52, 53). We realize that the prevalence of *p16* alterations may be underestimated in patients with very short BE segments, because fewer biopsies were taken in these patients. However, the expansion of clones with *p16* lesions to all or all but one levels of the Barrett's segment in 82% of the patients examined decreases the likelihood that increased sampling of larger segments affected the probability of detecting existing *p16* lesions.

Our results may also explain a recent report (54) that cyclin D overexpression in BE is a predictor of progression to esophageal adenocarcinoma. *p16* displaces cyclin D from the CDK complex that promotes cell division (5), thereby preventing entry into S-phase of the cell cycle (55, 56), and *p16* loss is associated with cyclin D overexpression in esophageal cancer cell lines (57). Thus, cyclin D overexpression may be a surrogate for *p16* inactivation in BE. Further work will be required to investigate this hypothesis as well as to determine whether cyclin D overexpression or *p16* lesions are the best predictors of progression in BE.

In summary, *p16* lesions are the earliest known somatic genetic/epigenetic abnormalities in BE occurring in >85% of cases at all histological grades of dysplasia. *p16* hemizygous clones are progenitors to *p16* nullizygous clones, and both undergo clonal expansion to involve large regions of the esophagus, creating a field in which other premalignant genetic lesions can arise that can result in esophageal adenocarcinoma.

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