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p16^{INK4a} Promoter Is Hypermethylated at a High Frequency in Esophageal Adenocarcinomas[1]

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Abstract

Loss of heterozygosity (LOH) of 9p21, which contains the *p16*^{INK4a} tumor suppressor gene locus, is one of the most frequent genetic abnormalities in human neoplasia, including esophageal adenocarcinomas. Only a minority of Barrett's adenocarcinomas with 9p21 LOH have a somatic mutation in the remaining *p16* allele, and none have been found to have homozygous deletions. To determine whether *p16* promoter hypermethylation may be an alternative mechanism for *p16* inactivation in esophageal adenocarcinomas, we examined the methylation status of the *p16* promoter in flow-sorted aneuploid cell populations from 21 patients with premalignant Barrett's epithelium or esophageal adenocarcinoma. Using bisulfite modification, primer-extension preamplification, and methylation-specific PCR, we demonstrate that the methylation assay can be performed on 2 ng of DNA (~275 cells). Eight of 21 patients (38%) had *p16* promoter hypermethylation and 9p21 LOH, including 3 patients who had only premalignant Barrett's epithelium. Our data suggest that promoter hypermethylation with LOH is a common mechanism for inactivation of *p16* in the pathogenesis of esophageal adenocarcinomas.

Introduction

The incidence of *p16* inactivation is reported to be second only to p53 inactivation in human neoplasia (1). *p16* was initially shown to be a target for 9p21 LOH,[3] and thus a tumor suppressor, by mutations and homozygous deletions in tumor cell lines (2), germ-line mutations in familial melanoma kindreds (3), and somatic mutations and homozygous deletions in pancreatic adenocarcinomas (4). However, in many primary tumors with 9p21 LOH, including esophageal adenocarcinomas, *p16* mutations or homozygous deletions are relatively rare (5, 6), suggesting that either a second tumor suppressor gene exists at this locus or that *p16* is inactivated by an alternative mechanism. At least two additional cell cycle regulators, *p15*^{INK4b} and *p16β*, are located at 9p21. *p15* has been reported to be inactivated in gliomas and leukemias (7, 8), but *p16β* has not yet been shown to be selectively inactivated in any human neoplasm (9). Thus, *p16* remains the predominant tumor suppressor gene on 9p21. An alternative mechanism for *p16* inactivation, promoter hypermethylation, was recently found to be a common event in a number of human cancers (10–12). Except in imprinted or X-inactivated genes, CpG island promoters are normally unmethylated (13). Hypermethylation of the *p16* CpG island promoter correlated with transcriptional silencing, whereas treatment with the demethylating agent, 5-deoxyazacytidine, reactivated transcription (10–12). Thus, multiple mechanisms for inactivation of the remaining *p16* allele in primary tumors with 9p21 LOH have been reported, but the frequency of each mechanism varies among tumor types (10–12, 14).

Barrett's esophagus is a condition in which a metaplastic columnar epithelium replaces the normal stratified squamous epithelium of the esophagus. This condition predisposes to the development of esophageal adenocarcinoma (15). 9p21 LOH is a highly selected abnormality in Barrett's esophagus, previously reported in 24 of 32 patients (75%) with premalignant Barrett's epithelium or esophageal adenocarcinoma (16). Furthermore, 9p21 LOH is an early lesion in the progression from Barrett's esophagus to adenocarcinoma, occurring before the evolution of aneuploid cell populations and cancer (16). However, mutations in the remaining *p16* allele were detected in only 5 of 22 patients (23%; Ref. 16). Homozygous deletions of *p16* have been reported in several types of primary tumors but were not detected in esophageal adenocarcinomas (4, 14, 16). Thus, we tested the hypothesis that the promoter of the remaining wild-type *p16* allele is hypermethylated.

Highly purified aneuploid cell populations from Barrett's premalignant epithelium and adenocarcinomas were isolated with DNA content flow cytometric cell sorting (17). Small quantities of DNA are a major limitation for assaying highly purified flow-sorted cells from human biopsy specimens. Previous approaches for assessing methylation status, using methylation-sensitive restriction enzymes with Southern hybridization or PCR, require large amounts of DNA and are prone to false positive results due to incomplete digestion. Bisulfite treatment of DNA, which converts all unmethylated but not methylated cytosines to uracil, has provided an effective alternative to restriction enzymes, but genomic sequencing of bisulfite-modified DNA is relatively labor-intensive and time-consuming (18). Recently, Herman *et al.* (19) developed an efficient PCR-based assay, methylation-specific PCR, that takes advantage of DNA sequence differences between methylated and unmethylated alleles after bisulfite modification by using primers that distinguish between the two types of alleles.

However, the amount of DNA required for methylation-specific PCR still precludes routine analysis of flow-sorted endoscopic biopsy samples from patients with Barrett's esophagus. We now report the ability to reduce the amount of DNA necessary for the assay at least 60-fold with PEP, a PCR-based method for whole-genome amplification using a mixture of degenerate 15-base oligonucleotide primers (20, 21). DNA extracted from flow-sorted biopsy samples was modified with bisulfite, amplified by PEP and then amplified with the methylation-specific primers, and finally, visualized on an agarose gel.

We assessed the *p16* methylation status of flow-sorted aneuploid populations from 21 patients who had Barrett's esophagus with premalignant epithelium or adenocarcinoma. Fourteen of 21 patients had 9p21 LOH without *p16* mutation, 5 patients had both 9p21 LOH and

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[3] The abbreviations used are: LOH, loss of heterozygosity; PEP, primer-extension preamplification.

p16 mutation, and 2 patients retained both alleles at 9p21. Our results demonstrate that hypermethylation of the *p16* promoter is a frequent abnormality in esophageal adenocarcinomas and that 9p21 LOH with *de novo p16* promoter hypermethylation is a common mechanism for the two-hit inactivation of *p16*.

Materials and Methods

Patient Tissue Samples, Flow Cytometric Sorting, and DNA Extraction. The Barrett's esophagus study was approved by the Human Subjects Division of the University of Washington in 1982 and renewed annually thereafter. Endoscopic and/or surgical tissue from 21 patients who had Barrett's esophagus with premalignant epithelium or adenocarcinoma was collected using mapping protocols that have been described previously (17). Aneuploid populations were isolated by DNA content flow cytometric cell sorting using a Coulter Elite Flow Sorter as described previously (17). Gastric tissue was obtained from each patient and used as a constitutive control. DNA from the flow-sorted aneuploid samples and the gastric samples was extracted by standard SDS-proteinase K treatment. All aneuploid populations were previously screened for 9p21 LOH and *p16* mutation (16).[4]

Bisulfite Modification. Bisulfite modification protocols (18, 19) were adapted for use with small quantities of DNA.[5] Briefly, 6 ng (~800 cells) of each DNA sample were denatured, in a final volume of 20 μl, in freshly prepared NaOH at a final concentration of 0.3 M for 20 min at 42°C. Freshly prepared 3.8 M sodium bisulfite (Sigma)/1.0 mM hydroquinone (Sigma) mixture (pH 5.0) was added to each denatured DNA sample to a final volume of 100 μl and incubated under mineral oil at 55°C for 6–8 h. DNA samples were then purified with the Wizard PCR Preps DNA purification resin (Promega), as specified by the manufacturer, and eluted in 50 μl of water, followed by treatment with NaOH at a final concentration of 0.3 M for 20 min at 37°C. Each DNA sample was purified by ethanol precipitation and resuspended in 20 μl of water.

PEP. Each PEP reaction contained the 20-μl bisulfite-modified DNA sample in a final volume of 60 μl. Reaction conditions used were described previously (20). All PCR reactions were performed using a MJ DNA Engine Tetrad Thermal Cycler (MJ Research, Inc.).

Methylation-specific PCR. Primer pairs for *p16* (*p16*-W, *p16*-M, and *p16*-U) and for *p15* (*p15*-W, *p15*-M, and *p15*-U) were used for methylation-specific PCR, as described previously (19). The PCR mixture included GeneAmp 10× PCR buffer (Perkin-Elmer Corp.), $MgCl_2$ (1.5 mM), deoxynucleotide triphosphates (200 μM), primer pair (10 pmol each/reaction), 10 μl of the PEP sample, and 1.25 units of AmpliTaq Gold (Perkin-Elmer Corp.) in a final volume of 25 μl. PCR reaction conditions used were as described previously (19), except for use of touchdown PCR for primer pairs *p16*-W and *p15*-W. Ten-μl aliquots of each allele-specific PCR sample were loaded onto a 2.5% agarose gel, stained with ethidium bromide, and visualized with Gel Documentation System 1000 (Bio-Rad).

Statistical Analysis. The prevalence of hypermethylation in different groups was compared using Fisher's exact test.

Results

We investigated whether PEP of bisulfite-modified DNA would be an accurate and efficient method by which we could reduce the starting amounts of DNA required for detection by methylation-specific PCR. To test the accuracy of methylation status results with the introduction of PEP into the protocol, we used the lung cancer cell lines H249, which is unmethylated at the *p16* and *p15* promoters, and H1618, which is methylated at both promoters (19). The methylation status results were identical with and without the use of PEP on the same bisulfite-treated samples of H249 and H1618 (data not shown). Without PEP, the methylation-specific PCR assay required bisulfite treatment of 120 ng of DNA (~16,000 cells) to investigate the methylation status of a single promoter region. We found that with PEP, 6 ng (~800 cells) of each DNA sample provided sufficient DNA to investigate the methylation status of three different loci. Thus, PEP enabled us to use 60-fold less DNA, without the need to design methylation-specific nested primers for each promoter region (22).

We examined the methylation status of the *p16* promoter in aneuploid populations from 21 patients with Barrett's esophagus that had been screened for 9p21 LOH. Nineteen of 21 patients had 9p21 LOH, and 5 of the 19 had *p16* mutations in the remaining allele (Table 1). The H249 and H1618 cell lines were used as positive controls for unmethylated and methylated alleles, respectively (Fig. 1, *A* and *B*). To test whether promoter hypermethylation may be an alternative mechanism for inactivation of the remaining *p16* allele, we initially investigated the prevalence of *p16* promoter hypermethylation in the 14 patients who had 9p21 LOH without *p16* mutation (Table 1, patients 1–14). *p16* promoter methylation of the remaining allele was found in 8 of 14 patients (57%; Table 1, patients 1–8; Fig. 1, *C* and *D*). Three patients had *p16* promoter hypermethylation in premalignant Barrett's epithelium without cancer (Table 1, patients 1–3). The remaining 6 of 14 patients who had 9p21 LOH without *p16* mutation were not hypermethylated at the *p16* promoter (Table 1, patients 9–14; Fig. 1*E*). None of the corresponding gastric samples had *p16* promoter hypermethylation (Fig. 1, *C–F*). The difference in prevalence of hypermethylation between Barrett's aneuploid cell populations and normal gastric tissues for these 14 patients is highly significant ($P = 0.002$). Thus, our data suggest that *p16* promoter hypermethylation is a *de novo* event that occurs at a high frequency in the pathogenesis of esophageal adenocarcinomas.

The five patients who had both 9p21 LOH and *p16* mutation were found to be unmethylated at the promoter of the remaining mutant *p16*

Table 1 *p16 promoter methylation status in aneuploid cell populations from premalignant Barrett's epithelium and adenocarcinoma*

Patient	DNA content	Neoplastic stage	*p16* hypermethylation
9p21 LOH/*p16* wt[a]			
1.[b] 17421[c]	3.1N[d]	pre[e]	+
2. 95931	3.2N	pre	+
3. 21631	4.3N	pre	+
4. 17031	3.5N	ca[f]	+
5. 13010	2.8N	ca	+
6. 00010	3.7N	ca	+
7. 92041	3.7N	ca	+
8. 73010	3.3N	ca	+
9. 43251	3.1N	pre	−
10. 50051	3.2N	pre	−
11. 02010	2.7N	ca	−
12. 02911	3.0N	ca	−
13. 87711	3.1N	ca	−
14. 71010	3.2N	ca	−
9p21 LOH/*p16* mut[g]			
15. 18431[h]	2.9N	pre	−
16. 69421[i]	3.0N	pre	−
17. 99041[i]	3.5N	pre	−
18. 92461[j]	3.3N	pre	−
19. 10010[j]	3.9N	ca	−
9p21 het[k]			
20. 51061	3.2N	pre	−
21. 46421	3.7N	ca	−

[a] wt, wild-type.
[b] Patient number.
[c] Patient code.
[d] N, ploidy.
[e] pre, premalignant.
[f] ca, cancer.
[g] mut, mutation.
[h] Codon 102, del 1.
[i] Codon 58, R → stop.
[j] Codon 80, R → stop.
[k] het, retention of both alleles.

[4] M. T. Barrett, unpublished data.
[5] R. Stöger, T. M. Kajimura, W. T. Brown, and C. D. Laird. Epigenetic variation illustrated by DNA methylation patterns of the fragile-X gene, FMR1, submitted for publication.

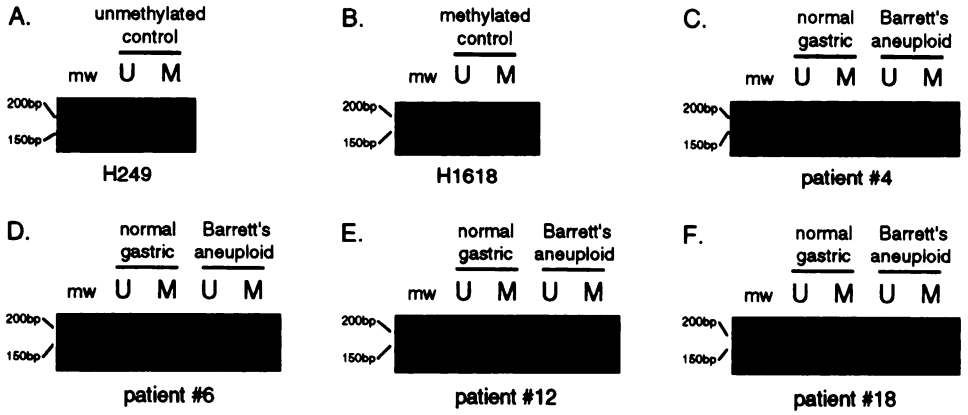

Fig. 1. Methylation-specific PCR analysis of *p16* promoter in normal tissues and aneuploid cell populations from patients with Barrett's esophagus. *mw*, 50-bp DNA ladder (Life Technologies, Inc.). Primer pairs used for amplification are designated as unmethylated (*U*) or methylated (*M*). *Patient numbers* refer to those in Table 1.

allele (Table 1, patients 15–19; Fig. 1*F*), resulting in a significant difference in the prevalence of hypermethylation in cases with and without *p16* mutation ($P = 0.04$). Neither of the two patients without 9p21 LOH was hypermethylated at the *p16* promoter (Table 1, patients 20 and 21). Thus, our results support the hypothesis that *p16* promoter hypermethylation is an alternative mechanism to *p16* mutation for inactivation of the remaining *p16* allele in cases with 9p21 LOH.

We also investigated whether *p15*, in addition to *p16*, may be a target for *de novo* hypermethylation in esophageal adenocarcinomas. In contrast to the *p16* promoter, the *p15* promoter was not hypermethylated in any of the 21 patients (data not shown). In the patients who had 9p21 LOH without *p16* mutation, the difference between the prevalence of *p16* hypermethylation (8 of 14) and *p15* hypermethylation (0 of 14) is highly significant ($P = 0.002$), suggesting that *p15* is not a selected target of 9p21 LOH in esophageal adenocarcinomas.

Discussion

Our study has provided evidence that *p16* may be inactivated in esophageal adenocarcinomas with 9p21 LOH by at least two different mechanisms: mutation or promoter hypermethylation. Nineteen of 21 patients with Barrett's esophagus (90%) had 9p21 LOH, and only 5 of these 19 patients (26%) had *p16* mutations in the remaining allele (Table 1). However, hypermethylation of the *p16* promoter, which previously has been correlated with transcriptional silencing and decreased promoter accessibility (10–12, 23), was found in 8 of 14 patients who had 9p21 LOH without *p16* mutation (57%; Table 1, patients 1–8). Overall, 8 of 21 (38%) patients had *p16* promoter hypermethylation, suggesting that *p16* promoter hypermethylation with 9p21 LOH is a common mechanism for inactivation of the *p16* gene in esophageal adenocarcinomas. Six patients had 9p21 LOH without *p16* mutation or promoter hypermethylation of the remaining allele (Table 1, patients 9–14), which may indicate a second tumor suppressor gene on 9p21 or an undetected *p16* mutation or deletion (24). Nevertheless, *p16* was selectively mutated or hypermethylated in 13 of 19 patients with 9p21 LOH (68%), suggesting that it is a primary target for inactivation during neoplastic progression in Barrett's esophagus.

Because the *p15* gene is linked to the *p16* gene at 9p21, the absence of *p15* promoter hypermethylation suggests that *de novo* hypermethylation is a selective abnormality at the *p16* promoter in Barrett's adenocarcinomas. *p15* previously has been shown to be selectively inactivated in gliomas and leukemias (7, 8). However, consistent with our previous mutational analysis of *p15* (16) and data from other primary tumors (2, 8, 25), the lack of *p15* promoter hypermethylation in esophageal adenocarcinomas suggests that, unlike *p16*, *p15* does not act as a tumor suppressor in Barrett's esophagus.

We have previously shown that both 9p21 LOH and *p16* mutation are early genetic abnormalities in the pathogenesis of esophageal adenocarcinomas, occurring in premalignant tissue before the evolution of aneuploidy and cancer (16, 26). The present study included 10 patients who had premalignant Barrett's epithelium without cancer. Nine of 10 premalignant aneuploid samples had 9p21 LOH, and 3 of these 9 had *p16* promoter hypermethylation (Table 1). The presence of *p16* promoter hypermethylation and 9p21 LOH in premalignant aneuploid cell populations suggests that *p16* inactivation is an early event in the progression from Barrett's esophagus to adenocarcinoma. However, additional studies need to be performed to determine whether *p16* hypermethylation occurs in premalignant diploid populations similar to 9p21 LOH and *p16* mutation.

Human neoplastic tissue samples are characterized by cellular heterogeneity, which often limits the ability to assay for somatic genetic lesions. Therefore, we use flow cytometric cell sorting, which permits the enrichment of highly purified cell populations. We have previously shown that DNA content flow cytometry purifies aneuploid cell populations to 99% or greater homogeneity, which allows unambiguous detection of LOH and mutations (17, 27). The absence of residual normal unmethylated alleles in aneuploid cell populations with promoter hypermethylation is consistent with the homogeneity of our flow-sorted biopsies (Fig. 1, *C* and *D*).

PEP of bisulfite-modified DNA is an accurate and efficient method to reduce the starting amounts of DNA required for methylation-specific PCR. Only 2 ng of DNA (~275 cells) are needed to determine the methylation status at one locus with primer pairs specific for the unmethylated and methylated alleles. The combination of PEP and methylation-specific PCR will be valuable in a variety of applications in which quantities of DNA are limited, such as in premalignant biopsies or in embryonic tissue. In addition, the use of PEP avoids the necessity of designing a set of nested primers for each locus in the genome, which will increase the efficiency of assessing methylation status at multiple loci.

In summary, we have demonstrated that *p16* promoter hypermethylation occurs at a high frequency in esophageal adenocarcinomas and that promoter hypermethylation with LOH is a common mechanism for the inactivation of *p16* in this cancer. We have also shown that *p16* promoter hypermethylation can occur in premalignant epithelium, analogous to 9p21 LOH and *p16* mutation. An important question will be to determine the stage of progression to Barrett's adenocarcinomas at which *p16* promoter hypermethylation occurs.

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