

Mutagen Sensitivity and Neoplastic Progression in Patients with Barrett's Esophagus: A Prospective Analysis

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Abstract

Background: Defects in DNA damage recognition and repair have been associated with a wide variety of cancers. We conducted a prospective study to determine whether mutagen sensitivity, as determined by an *in vitro* assay, was associated with the future development of cancer in patients with Barrett's esophagus, which is associated with increased risk of progression to esophageal adenocarcinoma.

Methods: We measured sensitivity to bleomycin in peripheral blood lymphocytes in a cohort of 220 patients with Barrett's esophagus. We followed these patients for 1,230 person-years (range, 3 months to 10.1 years; median, 6.4 years), using development of cancer and aneuploidy as end points. A subset of these patients was evaluated for inactivation of tumor-suppressor genes *CDKN2A/p16* and *TP53* [by mutation and loss of heterozygosity (LOH)] in their Barrett's segments at the time of, or before, the bleomycin test, and the patients

were stratified by *CDKN2A/p16* and *TP53* status in an analysis of mutagen sensitivity and progression.

Results: Bleomycin-sensitive patients were found to be at significantly greater risk of developing aneuploidy (adjusted hazard ratio, 3.71; 95% confidence interval, 1.44-9.53) and nonsignificantly greater risk of cancer (adjusted hazard ratio, 1.63; 95% confidence interval, 0.71-3.75). Among patients with detectable LOH at the *TP53* locus (on chromosome 17p), increasing bleomycin sensitivity was associated with increased risk of developing cancer ($P_{\text{trend}} < 0.001$) and aneuploidy ($P_{\text{trend}} = 0.005$).

Conclusions: This study supports the hypothesis that sensitivity to mutagens increases the risk of neoplastic progression in persons with Barrett's esophagus, particularly those with 17p LOH including *TP53*. (Cancer Epidemiol Biomarkers Prev 2006;15(10):1935-40)

Introduction

The incidence of esophageal adenocarcinoma has risen rapidly over the past 30 years in the United States and Western Europe (1-3). Most cases seem to arise in Barrett's esophagus, a metaplastic epithelium that develops in response to chronic gastroesophageal reflux disease (4-7). Although persons with Barrett's esophagus are at an elevated risk for progressing to esophageal adenocarcinoma, estimated at 0.5% to 1.0% per year (8-10), the vast majority of persons with Barrett's esophagus will not develop esophageal adenocarcinoma within their lifetimes. Thus, discrimination between persons at high risk of progression, who would benefit from more intensive prevention and surveillance programs, and those at relatively low risk, for whom lower-cost alternatives might be appropriate, is of critical importance. A variety of environmental and host factors are thought to play a role in the etiology of esophageal adenocarcinoma, including acid reflux (4, 5, 11-13), gender, race, obesity (14-18), *Helicobacter pylori* colonization, and cigarette smoking (14, 19, 20). The mechanisms of action of these factors are likely to directly or indirectly involve DNA damage. Increased levels of DNA damage have been detected in Barrett's mucosa (21) and may be associated with progression (22). Impaired ability to repair such damage may

therefore play a role in progression to esophageal adenocarcinoma, as has been suggested in a study of polymorphisms in DNA repair genes in persons with Barrett's esophagus (23).

The bleomycin mutagen sensitivity assay is an indirect measure of an individual's constitutive ability to repair DNA damage (refs. 24, 25; reviewed in refs. 26, 27). The assay measures the number of unrepaired bleomycin-induced double-strand chromatid breaks in peripheral blood lymphocytes *in vitro* and is believed to reflect the equilibrium between mutation rate and DNA repair (26). Lymphocytes from individuals with cancer, and in one study, oral premalignant lesions, have been found to exhibit higher bleomycin sensitivity than healthy controls (28-33). Bleomycin sensitivity may capture both individual susceptibility (27, 34-36) and environmental exposures, such as those to tobacco smoke (37) or oral selenium (36), although most studies have found that sensitivity is not affected by such exposures (38-40). Prospective studies of mutagen sensitivity and cancer risk are more difficult to conduct because the assay requires viable cells. Previous prospective bleomycin sensitivity studies have analyzed cohorts of patients with cancer and used the recurrence of cancer (41-43) or mortality (44) as end points.

Progression in Barrett's esophagus is associated with the inactivation of tumor-suppressor genes, in particular *CDKN2A/p16* [by mutation and chromosome 9p loss of heterozygosity (LOH)] and *TP53* (by mutation and 17p LOH; refs. 45, 46). Inactivation of these genes could allow cells with DNA damage to progress through the cell cycle, possibly increasing cancer risk for bleomycin-sensitive individuals. The *p16* tumor-suppressor gene is lost frequently and early during neoplastic progression in Barrett's esophagus. *p16* loss is associated with loss of the wild-type late G₁ arrest (47) and entry into cell cycle. *TP53* is a multifunction protein that mediates cell cycle arrest and apoptosis in response to a

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variety of conditions, including double-strand DNA breaks, hypoxia, and depleted nucleotide pools (48). Likewise, *TP53* mutation and 17p LOH occur frequently in esophageal adenocarcinoma and have been shown to predict progression (49-51). Inactivation of the tumor-suppressor pathways contributes to the extensive genetic instability that characterizes the development of esophageal adenocarcinoma (46, 52, 53). This instability can become manifest as DNA content abnormalities, such as aneuploidy or tetraploidy, detectable by flow cytometry. Flow cytometric abnormalities have been shown to be predictors of esophageal adenocarcinoma risk in Barrett's esophagus (53-55).

We therefore conducted a prospective study of persons with Barrett's esophagus to determine whether mutagen sensitivity is associated with the subsequent development of cancer and the intermediate end point of aneuploidy. To determine the effects of bleomycin sensitivity in patients with inactivated tumor-suppressor genes, we compared the rates of progression in the subsets of patients with and without detectable alterations in *p16* or *TP53* coincident with or before the bleomycin sensitivity test. Our results indicate that bleomycin-sensitive Barrett's esophagus patients are at significantly increased risk for developing aneuploidy and may be at increased risk of developing esophageal adenocarcinoma.

Materials and Methods

Study Subjects. Patients were enrolled in the Seattle Barrett's Esophagus Study, originally 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 Institutional Review Board from 1993 to 2001. Since 2001, the study has been approved by the Fred Hutchinson Cancer Research Center Institutional Review Board with reciprocity from the University of Washington Human Subjects Division. Endoscopic biopsies of Barrett's epithelium, acquired at 2-cm intervals in the esophagus according to a standard protocol (53, 55), were evaluated from 220 patients who had Barrett's esophagus without cancer at the time of the endoscopy associated with the blood draw for the bleomycin assay (the "baseline" endoscopy). Biopsies were evaluated by flow cytometry and sorted on the basis of proliferation/DNA content as described previously (56-58). All patients in this cohort received follow-up endoscopies (Table 1). Biopsies obtained at or before baseline from a subset of these patients were evaluated for 9p21 and 17p LOH using polymorphic microsatellite markers, as described previously (58, 59). One hundred eighty-three patients were evaluated for 9p LOH, 181 patients for 17p LOH, 178 for *p16* mutation, and 180 for *TP53* mutation.

Bleomycin Sensitivity Assay. A blood sample (10 mL) was obtained for all patients at the time of the baseline endoscopy. Blood was drawn into sodium heparinized tubes, packed on dry ice, and shipped overnight to the laboratory of Dr. Xifeng Wu in Houston, TX. One milliliter of whole blood was cultured in 9 mL RPMI 1640 tissue culture medium (JRM Biosciences, Lenexa, KS) supplemented with 20% fetal bovine serum and 1.25 (volume for volume) phytohemagglutinin (Wellcome Research Laboratories, Research Triangle Park, NC). At 67 hours, bleomycin (Nippon Kayaku Co., Ltd., Tokyo, Japan)

was added to each culture to a final concentration of 0.03 units/mL for 5 hours. During the last hour, cells were treated with 0.04 μ g/mL Colcemid to induce mitotic arrest. Cells were treated with hypotonic 0.07 mol/L KCl solution for 12 minutes, fixed, washed with freshly prepared Carnoy's fixative (methanol and acetic acid 3:1), and air-dried on wet slides. Prepared slides were coded and stained with Giemsa solution. From stained preparations of each sample, 50 metaphases were examined under oil immersion and breaks were counted and expressed as the average number of breaks per cell. Gaps or attenuated regions were disregarded. Patients with an average of over 0.6 double-strand breaks per cell (the median number in our study) were deemed to be bleomycin sensitive. We also used 0.8 breaks per cell as a threshold for bleomycin sensitivity, as previously defined in ref. 25.

Interview and Anthropometric Data. At the time of or before the baseline endoscopy, all 220 subjects underwent structured interviews carried out in person by trained staff as described in refs. (60, 61) to determine the use of tobacco, alcohol, and medications. Anthropometric measurements were taken at the time of this interview and follow-up visits using a standardized protocol.

Statistical Analysis. Kaplan-Meier curves were used to plot the cumulative incidence of aneuploidy and cancer. A proportional hazards model was used to calculate the hazard ratios (HR), 95% confidence intervals (95% CI), and *P* values. The HRs were adjusted as necessary using patient age (as a continuous variable), gender, nonsteroidal anti-inflammatory drug use (current/former/never), tobacco (ever/never), and waist-to-hip ratio (above/below gender-specific median) using the subset of 219 patients (out of 220) for whom we had information on all of these factors. Statistical analyses were carried out using the R statistical computing language version 2.3.0 (62).

Results

Patients were followed from 3 months to 10.1 years; patient follow-up is summarized in Table 1. Table 2 summarizes the characteristics and bleomycin sensitivity assay results for the 220 Barrett's esophagus patients in the cohort. The mean numbers of bleomycin-induced breaks were slightly but not significantly higher among males, persons over 70 years, those with lower waist-to-hip ratios, and current nonsteroidal anti-inflammatory drug users. The Kaplan-Meier curves describing cumulative incidence of cancer and aneuploidy stratified by bleomycin sensitivity are shown in Fig. 1.

Bleomycin-sensitive (having more than the median of 0.6 breaks per cell) patients had a statistically significantly greater risk of developing aneuploidy (adjusted HR, 3.71; 95% CI, 1.44-9.53, *P* = 0.006) and a nonsignificant 1.63-fold increased risk (95% CI, 0.71-3.75) of developing cancer (Table 3). The results were similar when the threshold for bleomycin sensitivity was raised from >0.6 to >0.8 or >1.0 [cutoffs commonly used in the literature (25); data not shown for the 1.0 cutoff] breaks per cell and when the interquartile (quartiles defined as <0.44 breaks per cell, $0.44 \leq$ breaks < 0.6, $0.6 \leq$ breaks < 0.87, and ≥ 0.87 breaks) HRs of increasing bleomycin sensitivity were compared (Table 3). Bleomycin sensitivity was not significantly associated with the future development of cancer when the number of bleomycin-induced breaks was modeled as a continuous variable (adjusted *P* = 0.23) or as an ordinal variable representing the bleomycin break quartiles (adjusted *P* = 0.24; see Table 3 for interquartile HRs).

We stratified the cohort based on the presence or absence of chromosome 9p (*p16*) LOH, 17p (*TP53*) LOH, *p16* mutation, and *TP53* mutation in the Barrett's segment either at or immediately before the endoscopy that coincided with the bleomycin sensitivity assay (Table 4). Among patients with detectable 9p or 17p LOH, bleomycin sensitivity (>0.6 breaks

Table 1. Distribution of follow-up by outcome

	<i>n</i>	No. events	No. person-years	Median follow-up, y (range)
Cancer	220	27	1,230	6.4 (0.2-10.1)
Aneuploidy	180	23	982	6.3 (0.2-10.1)

Table 2. Average number of bleomycin-induced breaks per patient at the time of the baseline endoscopy by selected characteristics (220 patients)

	No. persons	Percentage (%)	Bleomycin-induced DNA breaks (mean \pm SD)
Total	220	100.0	0.69 \pm 0.36
Gender			
Male	183	83.2	0.70 \pm 0.36
Female	37	16.8	0.65 \pm 0.38
Age (y)			
26-54	66	30.0	0.67 \pm 0.37
55-69	97	44.1	0.68 \pm 0.36
≥ 70	57	25.9	0.74 \pm 0.37
Tobacco use*			
Current	24	10.9	0.63 \pm 0.33
Former	123	55.9	0.70 \pm 0.39
Never	73	33.2	0.70 \pm 0.34
Waist-to-hip ratio*, [†]			
≤ 0.900	53	24.1	0.74 \pm 0.39
0.901-0.951	55	25.0	0.74 \pm 0.40
0.952-0.998	56	25.5	0.61 \pm 0.28
≥ 0.998	55	25.0	0.68 \pm 0.38
NSAID use*			
Current	78	35.5	0.72 \pm 0.39
Former	49	22.3	0.66 \pm 0.31
Never	93	42.3	0.68 \pm 0.37

Abbreviation: NSAID, nonsteroidal anti-inflammatory drug.

*Interview and anthropometric data were taken before baseline for some patients.

[†]The waist-to-hip ratio of one patient was not available.

per cell) was associated with a significantly greater risk of developing aneuploidy. Among patients with 17p LOH, bleomycin sensitivity approached significance for cancer outcome ($P = 0.053$), and we found a significant trend modeling the number of bleomycin-induced breaks as a

continuous variable. The HR from such a model comparing the third versus first quartiles (corresponding to 0.44 breaks) was 3.25 (95% CI, 1.62-6.53, $P_{\text{trend}} < 0.001$) for patients with 17p LOH and 1.20 (95% CI, 0.51-2.83, $P_{\text{trend}} = 0.68$) for patients without 17p LOH. The HR using aneuploidy as an end point is 35.5 (95% CI, 2.92-430, $P_{\text{trend}} = 0.005$) for patients with 17p LOH and 1.63 (95% CI, 0.85-3.11, $P_{\text{trend}} = 0.14$) for patients without 17p LOH. Bleomycin sensitivity was not a significant predictor of cancer in patients with (or without) 9p LOH, *TP53* mutation, or *p16* mutation.

Discussion

Defects in DNA damage recognition and repair have been associated with a wide variety of cancers (63). Barrett's esophagus is characterized by chronic inflammation, cellular damage/repair, and increased proliferation (56, 64-66). Chronic inflammation is associated with oxidative damage and increased levels of double-strand DNA breaks. Thus, diminished DNA repair in Barrett's esophagus could lead to accelerated progression to esophageal adenocarcinoma. Here, we report that Barrett's esophagus patients whose peripheral blood lymphocytes were sensitive to bleomycin-induced double-strand DNA breaks were at significantly increased risk for subsequent development of aneuploidy, a validated intermediate marker of progression to esophageal adenocarcinoma (53-55), and, to a lesser extent, esophageal adenocarcinoma itself.

Progression in persons with Barrett's esophagus is associated with increasing chromosomal instability (46). LOH at the *TP53* locus (17p LOH) generally precedes aneuploidy in persons with Barrett's esophagus (57, 67), and patients with 17p LOH are at increased risk for progression to esophageal adenocarcinoma (49). Although the bleomycin-sensitive patients in aggregate were not significantly more likely to progress to esophageal adenocarcinoma in our study, the risk among those with 17p LOH at or before baseline was significantly higher. We hypothesize that bleomycin-sensitive individuals have higher spontaneous chromosomal mutation rates and/or diminished DNA repair capacity, and, in that background, the loss of p53 function in the Barrett's epithelium allows cells to continue to cycle although chromosomal damage may not be fully

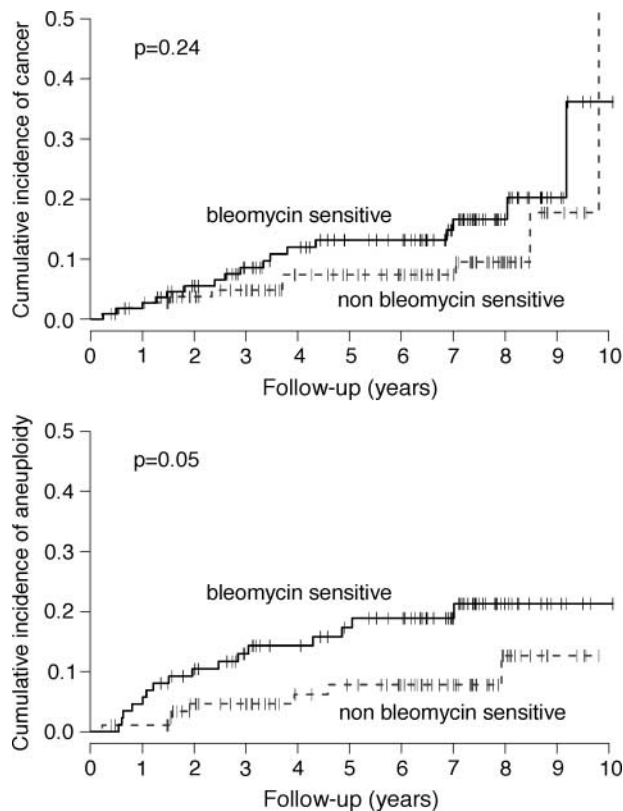


Figure 1. Kaplan-Meier curves for cancer and aneuploidy outcome for bleomycin-sensitive (>0.6 bleomycin-induced breaks per cell, solid lines) and nonsensitive (≤ 0.6 breaks per cell, dashed lines) patients.

Table 3. Crude and adjusted HR for cancer and aneuploidy by bleomycin sensitivity

No. breaks	Cancer						Aneuploidy					
	Events	<i>n</i>	Crude HR (95% CI)	<i>P</i>	Adjusted HR [†]	<i>P</i>	Events	<i>n</i>	Crude HR	<i>P</i>	Adjusted HR [†]	<i>P</i>
≤0.6	10	110	1.0*		1.0*		7	93	1.0*		1.0*	
>0.6	17	109	1.60 (0.74-3.51)	0.24	1.63 (0.71-3.75)	0.25	16	87	2.44 (1.00-5.93)	<0.05	3.71 (1.44-9.53)	0.006
≤0.8	16	155	1.0*		1.0*		12	129	1.0*		1.0*	
>0.8	11	64	1.86 (0.85-4.06)	0.12	1.74 (0.74-4.09)	0.20	11	51	2.41 (1.06-5.47)	0.04	4.02 (1.64-9.85)	0.002
<0.44	5	50	1.0*		1.0*		2	43	1.0*		1.0*	
≥0.44,<0.6	5	55	1.17 (0.33-4.09)	0.81	1.20 (0.34-4.23)	0.77	4	46	1.98 (0.36-10.8)	0.43	1.90 (0.34-10.54)	0.46
≥0.6,<0.87	8	59	1.31 (0.43-4.01)	0.64	1.44 (0.45-4.61)	0.54	7	47	3.19 (0.66-15.4)	0.15	4.11 (0.81-20.71)	0.09
≥0.87	9	55	2.03 (0.67-6.18)	0.21	1.98 (0.61-6.41)	0.25	10	44	5.33 (1.17-24.3)	0.03	10.72 (2.19-52.52)	0.003

NOTE: The first row of each set of comparisons summarizes the reference groups [patients with ≤0.6, ≤0.8, and in the first quartile of bleomycin breaks (<0.44 breaks per cell)], and the subsequent rows summarize the outcomes and HRs with respect to the reference groups.

*Reference group.

[†] HRs and 95% CIs are adjusted for age, gender (M/F), waist-to-hip ratio (low/high), tobacco use (never/ever), and nonsteroidal anti-inflammatory drug use (current/former/never). Waist-to-hip median was calculated with respect to gender.

repaired. G₁ arrest in cells with double-strand breaks is believed to be p53 dependent (68, 69), and there is strong evidence that alterations in a number of damage repair genes are associated with the development of cancer (70).

To our knowledge, ours is the first prospective study of bleomycin sensitivity (and perhaps mutagen sensitivity in general) and cancer risk before the onset of cancer. Because current technology does not allow us to measure the *in vivo* DNA repair capacity of individuals directly, we used the bleomycin assay as an indirect measure of the balance between DNA double-strand break formation and repair. A prospective study of mutagen sensitivity in cancer-free patients ensures that mutagen sensitivity is not influenced by the presence of cancer or its treatment. Detecting the association between bleomycin sensitivity and cancer risk in patients with 17p LOH was possible because of the frequent and long-term follow-up of the patients, the molecular characterization of our cohort using known biomarkers, and the relatively large number of cancer outcomes due to the increased risk of the cohort for

developing esophageal adenocarcinoma. Our use of aneuploidy, a known risk factor for developing esophageal adenocarcinoma, as an intermediate end point strengthened the study. However, some of our analyses were hindered by the limited number of cancer outcomes, especially when stratifying the cohort on molecular criteria. There may have been insufficient cancer outcomes to conclusively determine whether bleomycin sensitivity predicts esophageal adenocarcinoma in our cohort in aggregate, although the quartile trend in Table 3 is consistent with bleomycin sensitivity being associated with the future development of esophageal adenocarcinoma.

This study supports the hypothesis that sensitivity to mutagens can increase the risk of developing cancer, particularly among those with inactivated tumor-suppressor genes. We also find an association between an *in vitro* mutagen sensitivity assay and the development of aneuploidy in Barrett's esophagus epithelium *in vivo*. Further studies would be required to determine whether mutagen sensitivity assays could help risk-stratify patients with Barrett's esophagus.

Table 4. Crude HR for bleomycin sensitivity using cancer and aneuploidy end points stratified by tumor-suppressor mutation and LOH status

	Cancer				Aneuploidy			
	Events	<i>n</i>	Crude HR (95% CI)	<i>P</i>	Events	<i>n</i>	Crude HR (95% CI)	<i>P</i>
17p het								
≤0.6	3	68	1.0*		3	61	1.0*	
>0.6	5	80	1.33 (0.32-5.57)	0.70	9	67	2.61 (0.71-9.65)	0.15
17p LOH								
≤0.6	6	19	1.0*		2	10	1.0*	
>0.6	11	14	2.69 (0.99-7.31)	0.053	5	5	8.79 (1.68-46.1)	0.01
9p het								
≤0.6	3	30	1.0*		1	25	1.0*	
>0.6	2	37	0.39 (0.07-2.37)	0.31	4	33	2.92 (0.33-26.1)	0.34
9p LOH								
≤0.6	6	58	1.0*		4	47	1.0*	
>0.6	14	58	2.30 (0.88-5.99)	0.09	11	39	3.64 (1.15-11.5)	0.03
TP53 wt								
≤0.6	4	71	1.0*		5	65	1.0*	
>0.6	10	84	2.00 (0.63-6.38)	0.24	12	69	2.16 (0.76-6.14)	0.15
TP53 mut								
≤0.6	5	15	1.0*		0	6	1.0*	
>0.6	6	10	2.18 (0.66-7.18)	0.20	2	3	—	—
p16 wt								
≤0.6	7	73	1.0*		6	62	1.0*	
>0.6	12	76	1.50 (0.59-3.80)	0.40	13	60	2.21 (0.84-5.83)	0.11
p16 mut								
≤0.6	3	13	1.0*		0	10	1.0*	
>0.6	3	16	1.28 (0.21-7.65)	0.79	2	12	—	—

NOTE: Bleomycin sensitivity is defined as having >0.6 breaks per cell.

*Reference group.

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