Title: Mutagen sensitivity and neoplastic progression in patients with Barrett's esophagus: A prospective analysis

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Financial support: NIH PO1 CA91955, NIH R01 CA72030, NIH RO1 CA61202, NIH RO1 CA78828, NIH R01 CA119224-01, NIH T32 CA80416, NIH F32 CA84753, NIH DK07742, NIH K01 CA89267-02, and Commonwealth Universal Research Enhancement Program, Pennsylvania Department of Health

Running title: Mutagen sensitivity and neoplastic progression
Key words: mutagen sensitivity, epidemiology, Barrett's esophagus, esophageal adenocarcinoma

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 BE. 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 endpoints. 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 HR=3.71; 95% CI=1.44–9.53) and non-significantly greater risk of cancer (adjusted HR=1.63; 95% CI=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 (trend $p<0.001$) and aneuploidy (trend $p=0.005$).

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.

Introduction

The incidence of esophageal adenocarcinoma (EA) has risen rapidly over the past 30 years in the US and Western Europe [1–3]. Most cases appear to arise in Barrett's esophagus (BE), a metaplastic epithelium that develops in response to chronic gastroesophageal reflux disease (GERD) [4–7]. While persons with BE are at an elevated risk for progressing to EA, estimated at 0.5–1.0% per year [8–10], the vast majority of persons with BE will not develop EA 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 EA, 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 EA, as has been suggested in a study of polymorphisms in DNA repair genes in persons with BE [23].

The bleomycin mutagen sensitivity assay is an indirect measure of an individual's constitutive ability to repair DNA damage [24, 25] (reviewed in [26] and [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 pre-malignant 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 endpoints.

Progression in BE 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) [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 BE. p16 loss is associated with loss of the wild-type late G1 arrest [47] and entry into cell cycle. TP53 is a multi-function protein that mediates cell cycle arrest and apoptosis in response to a variety of conditions, including double-strand DNA breaks, hypoxia, and depleted nucleotide pools [48]. Likewise, TP53 mutation and 17p LOH occur frequently in EA and have been shown to predict progression [49–51]. Inactivation of the tumor suppressor pathways contribute to the extensive genetic instability that characterizes the development of EA [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 EA risk in BE [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 endpoint 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 prior to 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 EA.

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 (FHCRC) Institutional Review Board from 1993 to 2001. Since 2001, the study has been approved by the FHCRC IRB 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 BE 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 10-ml blood sample 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, Texas. 1 ml of whole blood was cultured in 9 ml of RPMI-I640 tissue culture medium (JRM Biosciences, Lenexa, KS) supplemented with 10% fetal calf serum and 0.2 ml of 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 μg/ml colcemid to induce mitotic arrest. Cells were treated with hypotonic 0.07 M 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 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/cell as a threshold for bleomycin sensitivity, as previously defined in [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 [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 (CI), and p values. The HRs were adjusted as necessary using patient age (as a continuous variable), gender, NSAID 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 BE patients in the cohort. The mean numbers of bleomycin-induced breaks were slightly but not significantly higher among males, persons over 70, those with lower waist-to-hip ratios, and current NSAID users. The Kaplan-Meier curves describing cumulative incidence of cancer and aneuploidy stratified by bleomycin sensitivity are shown in Figure 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 non-significant 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 inter-quartile (quartiles defined as <0.44 breaks/cell, $0.44 \leq$ breaks < 0.6, $0.6 \leq$ breaks < 0.87, and ≥ 0.87 breaks) hazard ratios 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 inter-quartile 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 per cell) was associated with a 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 vs. first quartiles (corresponding to 0.44 breaks) was 3.25 (95% CI=1.62–6.53, trend p<0.001) for patients with 17p LOH and 1.20 (95% CI=0.51–2.83, trend p=0.68) for patients without 17p LOH. The HR using aneuploidy as an endpoint is 35.5 (95% CI=2.92–430, trend p=0.005) for patients with 17p LOH and 1.63 (95% CI=0.85–3.11, trend p=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]. BE 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 BE could lead to accelerated progression to EA. Here, we report that BE 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 EA [53–55], and, to a lesser extent, EA itself.

Progression in persons with BE is associated with increasing chromosomal instability [46]. LOH at the TP53 locus (17p LOH) generally precedes aneuploidy in persons with BE [57, 67], and patients with 17p LOH are at increased risk for progression to EA [49]. Although the bleomycin-sensitive patients in aggregate were not significantly more likely to progress to EA 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 even though chromosomal damage may not be fully 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 cohort's increased risk for developing EA. Our use of aneuploidy, a known risk factor for developing EA, as an intermediate endpoint 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 EA in our cohort in aggregate, although the quartile trend in Table 3 is consistent with bleomycin sensitivity being associated with the future development of EA.

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 or not mutagen sensitivity assays could help risk-stratify patients with BE.

Acknowledgments

We thank David Cowan, Janine Kikuchi, and Terri Watson for database management; Christine Karlsen for coordination of patient care; Valerie Cerera for coordination of research biopsies and flow cytometry; Tricia Christopherson for management of patient interviews and data collection; and the study interviewers and participants in the Seattle Barrett's Esophagus Project for making this study possible.

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	n	number of events	number of person-years	medianfollow-up in years (range)
Cancer	220	27	1230	6.4 (0.2–10.1)
Aneuploidy	180	23	982	6.3 (0.2–10.1)

Table 1. Distribution of follow-up by outcome.

	# persons	%	bleomycin-induced DNA breaks (mean ± sd)
Total	220	100.0%	0.69 ± 0.36
Gender			
Male	183	83.2%	0.70 ± 0.36
Female	37	16.8%	0.65 ± 0.38
Age (years)			
26–54	66	30.0%	0.67 ± 0.37
55–69	97	44.1%	0.68 ± 0.36
≥70	57	25.9%	0.74 ± 0.37
Tobacco use[a]			
Current	24	10.9%	0.63 ± 0.33
former	123	55.9%	0.70 ± 0.39
never	73	33.2%	0.70 ± 0.34
Waist-to-hip ratio[a,b]			
≤0.900	53	24.1%	0.74 ± 0.39
0.901–0.951	55	25.0%	0.74 ± 0.40
0.952–0.998	56	25.5%	0.61 ± 0.28
≥0.998	55	25.0%	0.68 ± 0.38
NSAID use[a]			
Current	78	35.5%	0.72 ± 0.39
former	49	22.3%	0.66 ± 0.31
never	93	42.3%	0.68 ± 0.37

[a]Interview and anthropometric data taken before baseline for some patients.

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

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

	Cancer						Aneuploidy					
# breaks	events	n	crude HR (95% CI)	P	adjusted HR[a]	p	events	n	crude HR	p	adjusted HR[a]	p
≤ 0.6	10	110	1.0[b]		1.0[b]		7	93	1.0[b]		1.0[b]	
>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[b]		1.0[b]		12	129	1.0[b]		1.0[b]	
>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[b]		1.0[b]		2	43	1.0[b]		1.0b	
≥ 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

[a]HRs and 95% CIs adjusted for age, gender, waist-to-hip ratio, cigarette use, and NSAID use
[b]reference group

Table 3. Crude and adjusted HR for cancer (left columns) and aneuploidy (right columns) by bleomycin sensitivity. HR are adjusted for age, gender (M/F), waist-to-hip ratio (low/high), tobacco use (never/ever), and NSAID use (current/former/never). Waist-to-hip median was calculated with respect to gender. 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 hazard ratios with respect to the reference groups.

	Cancer				Aneuploidy			
	events	n	crude HR (95% CI)	p	events	n	crude HR (95% CI)	p
17p het								
≤ 0.6	3	68	1.0[a]		3	61	1.0[a]	
> 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[a]		2	10	1.0[a]	
> 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[a]		1	25	1.0[a]	
> 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[a]		4	47	1.0[a]	
> 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[a]		5	65	1.0[a]	
> 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[a]		0	6	1.0[a]	
> 0.6	6	10	2.18 (0.66–7.18)	0.20	2	3	—	—
p16 wt								
≤ 0.6	7	73	1.0[a]		6	62	1.0[a]	
> 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[a]		0	10	1.0[a]	
> 0.6	3	16	1.28 (0.21–7.65)	0.79	2	12	—	—

[a]reference group

Table 4. Crude HR for bleomycin sensitivity using cancer and aneuploidy endpoints stratified by tumor suppressor mutation and LOH status. Bleomycin sensitivity is defined as having >0.6 breaks per cell.

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