Significance of Crypt Atypia in Barrett's Esophagus: A Clinical, Molecular, and Outcome Study

Helen H. Wang,[1,2,*] Deepa T. Patil,[2,3,*] Thomas G. Paulson,[4] William M. Grady,[4,5] and Robert D. Odze[6]

[1]Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts; [2]Department of Pathology, Harvard Medical School, Boston, Massachusetts; [3]Department of Pathology, Brigham and Women's Hospital, Boston, Massachusetts; [4]Translational Science and Therapeutics Division, Fred Hutchinson Cancer Center, Seattle, Washington; [5]Department of Medicine, University of Washington School of Medicine, Seattle, Washington; and [6]Department of Pathology and Laboratory Medicine, Tufts University School of Medicine, Boston, Massachusetts

BACKGROUND & AIMS: **The aim of this study was to characterize baseline morphologic features of crypts in nondysplastic Barrett's esophagus and correlate them with DNA content abnormalities and risk of progression to high-grade dysplasia (HGD) or esophageal adenocarcinoma (EAC).**

METHODS: **The morphologic features of nondysplastic crypts in baseline biopsy specimens from 212 BE patients (2956 biopsy specimens) were graded histologically using a 4-point scale (crypt atypia levels, 0–3). DNA content abnormalities were detected using flow cytometry.**

RESULTS: **In patients who had dysplasia in their baseline biopsy specimens, dysplasia was associated significantly with increasing grades of crypt atypia in the background nondysplastic Barrett's esophagus ($P < .001$). In a subset of patients without dysplasia at baseline (N = 149), a higher grade of crypt atypia was associated with longer Barrett's esophagus segment length (5.5 vs 3.3 cm; $P = .0095$), and a higher percentage of cells with 4N DNA content (3.67 ± 1.27 vs 2.93 ± 1.22; $P = .018$). Crypt atypia was associated with the development of any neoplasia (low-grade dysplasia and HGD/EAC). Although no significant association was noted between the grade of crypt atypia and increased 4N, aneuploidy, or progression to HGD/EAC, only patients with grade 2 or 3 crypt atypia showed increased 4N, aneuploidy, or progression to HGD/EAC.**

CONCLUSIONS: **Patients with Barrett's esophagus likely develop dysplasia via a progressive increase in the level of crypt atypia before the onset of dysplasia, and these changes may reflect some alteration of DNA content.**

Keywords: Crypt Atypia; DNA Content; Barrett's Esophagus; Cancer Progression.

Barrett's esophagus (BE) is defined as columnar metaplasia of the esophagus, which is most often associated with intestinal metaplasia. BE is the main precursor to esophageal adenocarcinoma (EAC).[1] Cancer in BE develops via an inflammation-dysplasia-carcinoma sequence. Thus, patients with BE are advised to undergo regular endoscopic surveillance combined with biopsies to detect dysplasia and early cancer, which then potentially can be eliminated with endoscopic eradication therapy, such as endoscopic resection.[2] Dysplasia in its early stage can involve only the crypts and not the surface epithelium. Currently, cancer risk assessment is based entirely on morphologic identification and grading of dysplasia because they determine the risk of progression in the Barrett's segment. However, this is an imperfect risk-assessment system.

The Seattle biopsy protocol, which is the current gold standard for both screening and surveillance in patients with BE, unfortunately, suffers from several well-known deficiencies (eg, time consuming, poor adherence) that limit its overall efficacy as a method for prediction of neoplasia progression.[3] Furthermore, because dysplasia usually is focal and invisible, the Seattle protocol results in a relatively high degree of sampling error, and thus a high false-negative rate because it tends to grossly undersample the BE segment.[4–7] Prior studies have documented the presence of a wide variety of

*Authors share co-first authorship.

Abbreviations used in this paper: BE, Barrett's esophagus; CD, crypt dysplasia; EAC, esophageal adenocarcinoma; GI, gastrointestinal; HGD, high-grade dysplasia; LGD, low-grade dysplasia; NDBE, nondysplastic Barrett's epithelium.

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differentiation, proliferation, and even molecular abnormalities in nondysplastic epithelium in patients either with or without dysplasia in the setting of BE. These include alterations in cell cycle, DNA content, and even tumor-suppressor genes, such as *TP53*, among others.[8–10] Unfortunately, currently there are no data regarding the morphologic changes in the epithelium associated with any of these aforementioned abnormalities, and no data on whether there are phenotypic alterations that can be seen in the early stages of BE development, before the onset of dysplasia, that can help predict cancer progression. Therefore, identifying morphologic and/or molecular changes in nondysplastic Barrett's epithelium (NDBE) that can serve as markers of progression to high-grade dysplasia (HGD)/EAC, or of concurrent neoplasia in adjacent (unsampled) mucosa, will help overcome some of the known limitations of the Seattle protocol biopsy-based method of surveillance in BE.

Although it is well known that Barrett's "specialized" epithelium is characterized by a variety of "atypical" changes that render its appearance distinct from normal intestinal epithelium, the spectrum of changes that occur, and their possible correlation with risk of cancer development, have never been investigated. These include, at one end of the spectrum, epithelium with little to no "atypia" where the crypts are lined by cells with small normochromic nuclei without stratification or mitosis, to epithelium at the other end of the spectrum that shows marked "atypia" characterized by cells with nuclear hyperchromasia, enlargement, irregularity, stratification, and prominent mitoses. In the extreme, these crypt changes mimic the features of true dysplasia, and this has been termed *crypt dysplasia* (CD) in prior studies.[11,12] Thus, the purpose of this long-term outcome study was to evaluate and categorize the baseline crypt morphologic features of nondysplastic epithelium in BE, and to correlate these findings with DNA content abnormalities and risk of progression to cancer.

What You Need to Know

Background

Nondysplastic Barrett's epithelium can harbor a variety of molecular alterations and DNA content abnormalities. The crypts in nondysplastic epithelium of Barrett's esophagus (BE) patients show a spectrum of cytoarchitectural changes that range from glands with no atypia to crowded glands with significant atypia (previously described as crypt dysplasia).

Findings

Patients with dysplasia in their baseline biopsy specimens show a positive correlation between dysplasia and increasing grade of crypt atypia in the background nondysplastic Barrett's epithelium. In patients without dysplasia at baseline, a higher grade of crypt atypia is associated significantly with a longer BE segment and a higher percentage of cells with a 4N DNA content.

Implications for patient care

BE patients likely develop dysplasia via a progressive increase in the level of crypt atypia before the onset of dysplasia, and these changes may reflect some alteration of DNA content.

Methods

Clinical Design

The study included 212 patients with BE, all of whom were enrolled and participated in the Seattle Barrett's Esophagus Study. This was a prospective cohort of approximately 900 BE patients that began on July 1, 1983, and continued until January 30, 2013. All patients had a diagnosis of BE at entry into the study and were part of the prospective Seattle BE cohort surveillance program. There were no patients who had a new diagnosis of BE at the time of the index esophagogastroduodenoscopy. As part of this clinical and research protocol, all patients had clinically and pathologically confirmed BE according to published guidelines, no history of cancer, and had a baseline endoscopy and at least 1 prospective follow-up endoscopic and biopsy evaluation. At the time of the last (outcome) endoscopy, patients were categorized as either progressors (N = 41) if they had HGD (from NDBE or low-grade dysplasia [LGD] on index biopsy) or EAC (from NDBE, LGD, or HGD on index biopsy) documented histologically, or as nonprogressors (N = 171) if they did not. Of note, we included patients who were either negative for dysplasia or had LGD or HGD identified in their index specimens (the entire cohort). The study was approved by the University of Washington Human Subject Division in 1982 and renewed annually thereafter with reciprocity from the Internal Review Board of the Fred Hutchinson Cancer Center from 1993 to 2001. Since 2001, the study has been approved annually by the Institutional Review Board of the Fred Hutchinson Cancer Center with reciprocity from the Human Subject Division of the University of Washington.

Histology Methods

The presence or absence, grade of dysplasia, and morphologic features of the background nondysplastic crypts (evaluated according to a novel 4-point grading system developed by the authors) (Supplementary Table 1 and Figure 1) were analyzed by a gastrointestinal (GI) pathologist (R.D.O.) who was blinded to the flow cytometric and outcome results. The morphologic alterations were assessed in the basal portions of the

crypts and only in areas of mucosa without active inflammation and/or ulceration. The pathologist had no information about the presence or absence of dysplasia elsewhere in the BE segment when evaluating basal crypt atypia. All of these features were recorded in the mucosal biopsies from the patient's initial (baseline) endoscopic procedure only. The highest level of basal crypt atypia recorded in any of the baseline mucosal biopsy specimens served as the final crypt atypia grade for each patient. Dysplasia was graded according to previously published criteria.[13] A maximum neoplasia diagnosis was assigned for each biopsy and then for each patient. Outcome biopsy specimens were evaluated to confirm the presence or absence of HGD/EAC. The Supplementary Methods section lists the details for the endoscopy biopsy procedure, DNA content, and flow cytometry analysis.

Statistical Analysis

Comparison of continuous variables was performed with either the *t* test or the nonparametric Kruskal–Wallis test, depending on the sample size. Comparison of categoric variables was performed with either the chi-square or the Fisher exact test, again depending on the sample size. A 2-sided or 1-sided *P* value of $<.05$ was considered statistically significant. The risk ratio was defined as the risk of developing EAC during the study period in a particular group, such as those with low- or high-grade dysplasia, when compared with the risk of EAC in those without the particular characteristic, such as those without dysplasia. All statistical analyses were performed with STATA software (release 11; StataCorp LP, College Station, TX).

Results

Table 1 summarizes the patient characteristics in this study. A total of 212 deidentified patients with 2956 biopsy specimens who had complete data were included in this study. There were 168 men and 44 women, with an average age of 63 years at the time of enrollment in the Seattle BE study. The mean follow-up time interval was 91 months (range, 2.4–178 mo), with a mean of 14 biopsy specimens for each patient (range, 1–64 biopsy specimens). The mean length of the BE segment was 5.7 cm, and 63 of 212 (30%) had dysplasia of any grade present at their index endoscopy (LGD, N = 30; HGD, N = 33). Aneuploidy was present in 25 (12%) patients overall. The mean percentage of cells with a 4N DNA content was 4.37. Twenty-five patients had increased 4N (>6.0%). Overall, 41 patients (19%) progressed to HGD/EAC in the follow-up period and 171 did not. No significant differences were observed in any of these characteristics between men and women.

Overall, as expected, patients who progressed to HGD/EAC had a significantly higher proportion of index biopsy specimens with dysplasia compared with those

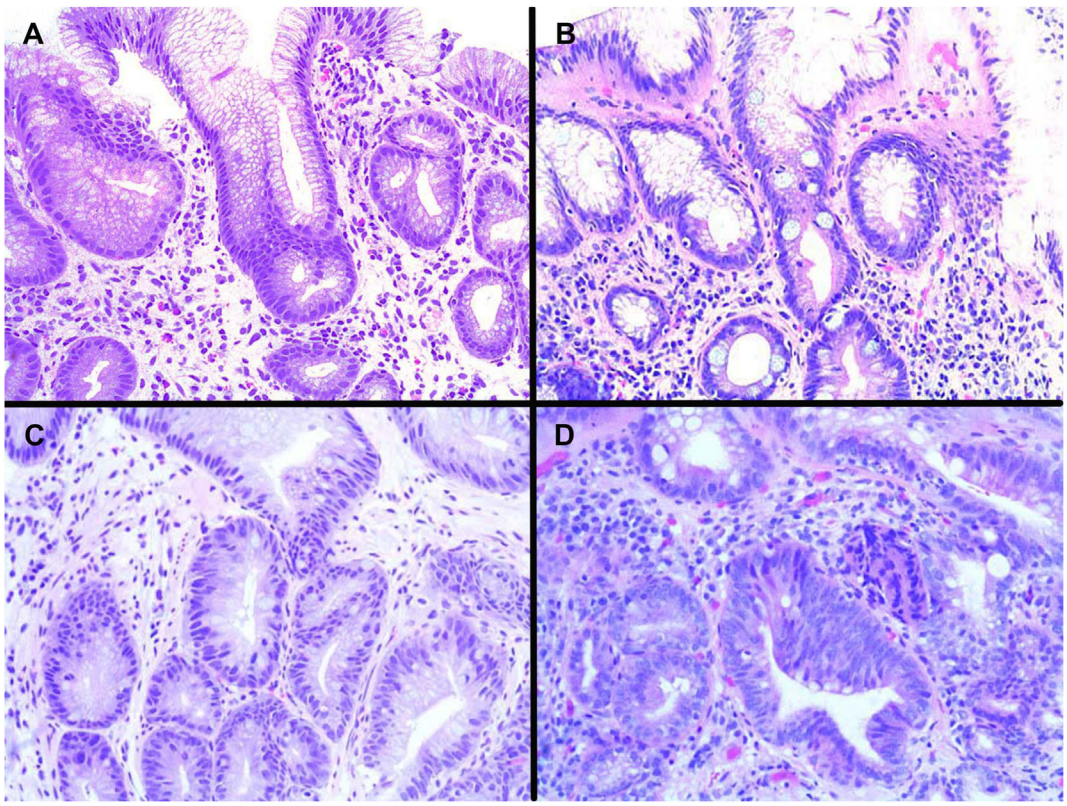

Figure 1. (*A*) Grade 0 atypia. (*B*) Grade 1 atypia. (*C*) Grade 2 atypia. (*D*) Grade 3 atypia.

who did not progress (31 of 41 [76%] vs 32 of 171 [19%]; $P < .001$). Thus, prevalent dysplasia on the index biopsy specimen was a highly significant predictor of progression to EAC. This difference primarily was owing to a significantly higher frequency of HGD in index biopsy specimens from patients who progressed compared with nonprogressors (23 of 41 [56%] vs 10 of 171 [5.8%]; $P < .001$). The progression rate showed a significant trend, with 6.7% among NDBE patients to 27% among those with LGD to 70% among those with HGD in index biopsy specimens ($P < .001$). The risk ratio for patients with LGD to develop EAC in the study period was 3.0 (95% CI, 0.75–12), and for those with HGD was 21 (95% CI, 8.5–51). Compared with nonprogressors, progressors had a significantly higher percentage of cells with a 4N DNA content (7.41 ± 5.44 vs 3.64 ± 1.43; $P < .0001$), increased 4N (39% vs 5.3%; $P < .001$), and aneuploidy (37% vs 5.8%; $P < .001$).

Table 2 summarizes the morphologic findings in the patient's index endoscopy, and the relationship between dysplasia and the maximum grade of crypt atypia in nondysplastic regions of BE mucosa, in patients with dysplasia. In the nondysplastic areas of BE mucosa, overall, 2 (0.94%) patients had grade 0 atypia, 20 (9.4%) patients had grade 1 atypia, 91 (43%) patients had grade 2 atypia, and 99 (47%) patients had grade 3 crypt atypia. There was a strong statistically significant relationship ($P < .001$) between dysplasia and an increasing degree of crypt atypia in the background nondysplastic epithelium. For instance, grade 3 crypt atypia was present in 87% and 79% of patients with LGD and HGD, respectively, but it was found in only 32% of patients without dysplasia. None of the patients with dysplasia had grade 0 crypt atypia and only 3 (9.1%) patients had grade 1 atypia in their background nondysplastic epithelium.

To determine the significance of the grade of crypt atypia independent of dysplasia, we evaluated, separately, the association between this feature and the other clinical, molecular, and outcome findings only in patients who did not have dysplasia identified in their baseline biopsy specimens (N = 149 patients). These results are summarized in Table 3. The grade of crypt atypia did not correlate with the mean age or gender of the patients. However, a higher grade of crypt atypia (2 or 3 vs 0 or 1) was associated significantly with longer BE segment length (5.5 vs 3.3 cm; $P = .0095$), and a higher percentage of cells with a 4N DNA content (3.67 ± 1.27 vs 2.93 ± 1.22; $P = .018$). No significant association was noted between the grade of crypt atypia and increased 4N, the presence of aneuploidy, or progression to HGD/EAC. However, only patients with either grade 2 or 3 crypt atypia showed increased 4N (N = 5), aneuploidy

Table 1. Patient Characteristics

Variable	Characteristics			
	All (n = 212)	Nonprogressors (n = 171)	Progressors to HGD or cancer (n = 41)	*P* value comparing nonprogressors with progressors
M:F ratio[a]	3.8:1 (168:44)	3.5:1 (133:38)	5:8 (35:6)	.28 by chi-square
Mean age, *y* (range)	63 (34–87)	63 (34–87)	64 (36–82)	.62 by *t* test
Mean number of biopsy specimens per patient (range)	14 (1–64)	11 (1–38)	25 (4–64)	<.0001 by *t* test
Length of BE, *cm* (range)	5.7 (0–20)	5.1 (0–20)	8.1 (0–19)	<.0001 by *t* test
No dysplasia on index endoscopy[b]	149	139 (93%)	10 (6.7%)	<.001 by chi-square
Any dysplasia on index endoscopy	63	32 (51%)	31 (49%)	<.001 by chi-square[c]
Low grade	30	22 (73%)	8 (27%)	.001 by chi-square[d]
High grade	33	10 (30%)	23 (70%)	<.001 by chi-square[e]
Percentage of cells with a 4N DNA content	4.37 ± 3.08	3.64 ± 1.43	7.41 ± 5.44	<.0001 by *t* test
Increased 4N (>6.0%)	25 (12%)	9 (5.3%)	16 (39%)	<.001 by chi-square
Aneuploidy present	25 (12%)	10 (5.8%)	15 (37%)	<.001 by chi-square
Follow-up period, *mo*	91 (2.4–178)	102 (2.4–178)	48 (3.0–155)	<.0001 by *t* test

BE, Barrett's esophagus; HGD, high-grade dysplasia; M:F, male:female.
[a]Raw/actual numbers of male and female subjects are indicated in parenthesis.
[b]Patients with no dysplasia on index endoscopy biopsy specimen is the reference category for comparison with the progression rate of those with any dysplasia, low-grade dysplasia, and high-grade dysplasia on the index endoscopy biopsy specimen.
[c]*P* value for comparison of the progression rate in patients with any dysplasia on the index biopsy specimen with patients with no dysplasia on the index biopsy specimen is <.001 by chi-square (49% vs 6.7%).
[d]*P* value for comparison of progression rate in patients with low-grade dysplasia on the index biopsy specimen with patients with no dysplasia on index biopsy specimen is <.001 by chi-square (27% vs 6.7%).
[e]*P* value for comparison of progression rate in patients with high-grade dysplasia on the index biopsy specimen with patients with no dysplasia on index biopsy specimen is <.001 by chi-square (70% vs 6.7%).

(N = 6), or progression to HGD/EAC (N = 10). None of the patients with grade 1 or 2 crypt atypia had any of these findings. In a subanalysis, a similar trend was observed between the degree of baseline crypt atypia and LGD as the final outcome variable. Only patients with grade 2 (6 of 83; 7.2%) or grade 3 atypia (11 of 47; 23%) progressed to LGD during the follow-up period compared with none (0%) of the grade 0 or grade 1 cases ($P = .13$). However, a significant association was noted between the presence of grade 2 or 3 baseline crypt atypia and the development of any neoplasia (either LGD or HGD/EAC) upon outcome (27 of 130; 21%), vs none (0%) among those with grade 0 or 1 atypia ($P = .02$).

Discussion

Patients with NDBE show a spectrum of cytologic and architectural features of the metaplastic epithelium. We performed this study to investigate the biological significance of the degree of crypt atypia present in nondysplastic epithelium in BE patients, and to determine its relationship to DNA content abnormalities and risk of progression to HGD/EAC. In this study of 2956 biopsy specimens from a well-defined, prospectively collected, longitudinal cohort of 212 BE patients, 41 of whom eventually progressed to HGD/EAC after long-term follow-up evaluation, we confirmed that patients with any dysplasia on their index biopsy specimens had a significantly higher progression rate to HGD/EAC (6.7% vs 49%; $P < .001$), which is consistent with multiple studies that have been published regarding prevalent dysplasia being a significant predictor of progression to cancer in BE. Using a novel grading system to evaluate the crypt morphologic alterations of nondysplastic epithelium in BE, we found a significant correlation between conventional dysplasia and crypt atypia in the background nondysplastic epithelium. In a further subanalysis of the BE patients without dysplasia at baseline, we found a positive correlation between grade of crypt atypia, and both BE segment length and the percentage of cells with a 4N DNA content, both as a continuous variable and dichotomized into increased (>6.0%) or not increased (≤6.0%), as determined by flow cytometry. Although only patients with either grade 2 or 3 crypt atypia showed increased 4N, aneuploidy, or progression to HGD/EAC, the association was not statistically significant. Based on these results, we conclude that patients with BE likely develop dysplasia via a progressive increase of crypt atypia before the onset of conventional dysplasia, and that these changes may reflect some alteration of DNA content within the cells. Although our progression results suggest that the level of crypt atypia may be associated with cancer risk, further studies using larger numbers of patients will need to be performed to determine if this hypothesis can be confirmed.

Pathologists have long observed that BE mucosa is composed of a heterogeneous population of cell types and glands that differ from normal intestinal crypts. Unfortunately, until now, few studies have focused on the background nondysplastic epithelium in BE and its potential biological and clinical significance. In 2010, Lomo et al[11] characterized the molecular features of CD. The histologic features of CD in that study corresponded to crypt atypia level 3 in our current study. In that study, 44% of patients with CD showed 17p (*TP53*) loss of heterozygosity compared with only 10% of patients without CD ($P = .016$). Furthermore, in that study, compared with adjacent nondysplastic, non-CD areas of epithelium, CD showed significantly increased p53 positivity (60% vs 13%; $P = .02$) and total crypt and basal crypt MIB-1 proliferation rate ($P < .001$). This was, essentially, the first study that suggested that dysplasia likely begins in the crypt bases and that these changes can be recognized and evaluated pathologically and are significant biologically in the metaplasia-dysplasia-carcinoma sequence in BE. Other more recent studies have confirmed that several genetic and epigenetic alterations may be detected in nondysplastic BE as well,[8–10] such as *TP53* alterations in NDBE. In a recent study, abnormal p53 expression was assessed in a retrospective cohort of 358 NDBE biopsy specimens and in a prospectively validated cohort of 646 NDBE biopsy

Table 2. Relationship Between Maximum Grade of Dysplasia and Degree of Crypt Atypia in Nondysplastic Area

	Degree of crypt atypia in nondysplastic area (% of total in that maximum grade of dysplasia)				
Maximum grade of dysplasia	0	1	2	3	Total
None	2 (1.3)	17 (11)	83 (56)	47 (32)	149
Low-grade	0	0	4 (13)	26 (87)	30
High-grade	0	3 (9.1)	4 (12)	26 (79)	33
Total	2 (0.94)	20 (9.4)	91 (43)	99 (47)	212

NOTE. The association between overall grade of dysplasia and degree of basal crypt atypia in nondysplastic areas was statistically significant by chi-square and the Fisher exact test with $P < .001$.

Table 3. Relationship Between Degree of Crypt Atypia and the Clinical and Molecular Features, and Outcome, in Patients Without Dysplasia on Their Index (Baseline) Biopsy (n = 149)

Variable	Degree of crypt atypia				*P* value comparing atypia 0 + 1 vs 2 + 3
	0 n = 2	1 n = 17	2 n = 83	3 n = 47	
Mean age, *y* (range)	61 (43–87)		61 (34–82)		.89 by *t* test
M:F ratio[a]	2.8:1 (14:5)		3.3:1 (100:30)		.76 by chi-square
Mean number of biopsy specimens per patient (range)	4.0 (2–6)	5.3 (1–16)	10 (2–38)	16 (2–43)	.0001 by *t* test
	5.2 (1–16)		12 (2–43)		
Mean length of BE, *cm* (range)	3.5 (3–4)	3.2 (1–8)	4.7 (0–20)	6.8 (0–15)	.0095 by *t* test
	3.3 (1–8)		5.5 (0–20)		
Percentage of cells with a 4N DNA content	2.65 ± 1.06	2.96 ± 1.26	3.57 ± 1.21	3.83 ± 1.36	.018 by *t* test
	2.93 ± 1.22		3.67 ± 1.27		
Increased 4N (>6.0%) (% of total in that degree of atypia)	0	0	3 (3.6)	2 (4.3)	.50 by 1-sided Fisher exact test
	0		5 (3.8)		
Aneuploidy present (% of total in that degree of atypia)	0	0	4 (4.8)	2 (4.3)	.34 by 1-sided Fisher exact test
	0		6 (4.6)		
Follow-up period, *mo*	124 ± 44	113 ± 53	103 ± 53	96 ± 54	.29 by *t* test
	115 ± 52		101 ± 53		
Outcome of HGD or cancer (% of total in that degree of atypia)	0	0	6 (7.2)	4 (8.5)	.36 by 1-sided Fisher exact test
	0		10 (7.7)		

BE, Barrett's esophagus; HGD, high-grade dysplasia; M:F, male:female.
[a]Raw/actual numbers of male and female subjects are indicated in parenthesis.

specimens. The authors found a strong correlation between abnormal p53 immunostaining in NDBE biopsy specimens and neoplastic progression.[14] Epigenetic changes, such as promoter hypermethylation in *APC*, *CDKN2A*, *MGMT*, and *TIMP3*, also have been observed in nondysplastic epithelium in BE.[15] A previous study by Liu et al[16] used image cytometry to evaluate DNA content abnormalities in NDBE epithelium, with and without goblet cells, and found that G0/G1 peak DNA index, DNA content heterogeneity index, and the percentage of cells with DNA exceeding 5N were significantly higher in epithelium containing goblet cells compared with normal gastric mucosa controls, and occur at an equal frequency and extent in metaplastic columnar epithelium of the esophagus without goblet cells, which suggests that metaplastic nongoblet columnar epithelium of the esophagus may have neoplastic potential. Our finding of an association between the level of crypt atypia and high percentage of cells with a 4N DNA content also suggests that there is a progressive sequence of DNA abnormalities in the preneoplasia stage of BE. Furthermore, although we did not observe a statistically significant association between the level of crypt atypia and other DNA abnormalities, such as aneuploidy and tetraploidy, it is interesting that these abnormalities were present only in patients with crypt atypia level 2 or 3, but not crypt atypia level 1. The lack of significance may be owing to type II statistical error, and this would need to be evaluated in a larger number of patients. However, it also is possible that these DNA alterations simply occur at a later stage of neoplastic progression in BE, and in fact there is evidence to support that hypothesis. For instance, prior studies have shown that the frequency of DNA aneuploidy increases with increasing histologic grades of dysplasia.[8,17–21] In a recent study, DNA content abnormality was identified in 95% of HGD, 21.1% of LGD, 9.5% of indefinite for dysplasia, and in none of the NDBE samples. In addition, patients with DNA content abnormality detected at baseline LGD or indefinite for dysplasia had a higher risk of subsequent detection of HGD or EAC.[21]

Ours is the first study to evaluate risk of neoplastic progression in BE patients with various levels of crypt atypia in a systematic manner. In our study, although we were unable to show a statistically significant correlation between the degree of crypt atypia and progression to

HGD/EAC, it is interesting to note that only those patients who harbored grade 2 (6 of 83 patients) or 3 (4 of 47 patients) atypia developed HGD/EAC. At least 2 prior studies (1 published only in abstract form) have evaluated long-term outcome among BE patients with level 3 crypt atypia, which also is termed CD, as discussed earlier. In a more recent retrospective study of 4545 patients who underwent wide-area transepithelial sampling with 3-dimensional computer-assisted analysis at 2 time points separated over a period of 12 months or more, progression in patients with baseline CD (grade 3 crypt atypia) was found to be significantly higher compared with those with baseline NDBE (and without CD) (1.42% per patient year vs 0.08% per patient year).[22] Interestingly, the concept that dysplasia in the GI tract may begin in the bases of the crypts and not involve the surface epithelium is, in fact, not novel because it has been described in other areas of the GI tract as well.

This study had several limitations that need to be highlighted. For instance, only 19 patients had either grade 0 or 1 crypt atypia compared with 83 with grade 2 and 47 with grade 3 atypia in patients without dysplasia on their index biopsy specimen. This is likely because the prospective Seattle BE cohort is a high-risk BE cohort, and, as such, it may be expected to be weighted toward higher levels of baseline crypt atypia and may not be representative of the general population with BE. Thus, this cohort of patients may have a higher likelihood of containing histologic and molecular alterations compared with the general BE population. Although this finding decreases the specificity of crypt atypia grade as a potential marker of progression, alternatively, it may help identify patients with grade 0 or 1 crypt atypia who have undergone extensive sampling at baseline and thus may be at very low risk of neoplastic progression. We also did not quantitate the degree (quantity) of crypt atypia in patients, but instead we categorized patients based on their highest crypt atypia grade identified in any of their biopsy specimens. Our rationale for this was based on the biology of neoplasia development in BE, where it is known that neoplastic changes in BE usually progress in a step-wise fashion via an underlying field effect, and on the principles of patient management, which is dependent on the patient's highest grade of dysplasia in any of the biopsy specimens.

Another potential limitation was that the specific biopsy specimens that were evaluated histologically for grading crypt atypia were not the ones specifically evaluated by flow cytometry, but they were obtained from the same anatomic level within the esophagus. All of the biopsy specimens were reviewed and graded by an expert GI pathologist who was blinded to the molecular and clinical outcome data. We did not perform an interobserver assessment of our novel grading system of crypt atypia primarily because our purpose was not to evaluate crypt atypia for clinical practice, but to better understand the biology of crypt atypia as it relates to molecular abnormalities and risk of neoplastic progression. As with other novel histology-based grading systems, this scale needs to be tested in the future for accuracy and reproducibility among pathologists if the data presented here are validated by others and a potential use in clinical practice is identified. Lastly, a limitation to this and, in fact, mostly all cohort studies of BE, is the lack of information on the duration of disease before enrollment in our study.

Our study also had some noteworthy strengths. For instance, we had long-term follow-up evaluation for all of our patients, it was a prospectively designed study, and included an extensive and standardized sampling protocol for all patients. Lastly, our study used DNA flow cytometric analysis with high-quality DNA content histograms to maximize the accuracy of interpreting and detecting DNA content abnormalities. Unfortunately, we were unable to perform histologic analysis on the same biopsy specimen that was used for flow cytometry analysis because the technique currently in use for that type of analysis had not yet been developed. Future studies that use selective analysis of macroscopically dissected atypical cells will be helpful to better characterize these DNA-level alterations.

In summary, in this study, we characterized the background morphologic features of nondysplastic epithelium in BE patients and provide evidence that the level of crypt atypia of the nondysplastic epithelium in BE is associated positively with conventional dysplasia and correlates with other risk factors of neoplastic progression as well, such as length of BE. We also found a significant correlation between increasing degrees of crypt atypia and one aspect of DNA content: the percentage of cells with a 4N DNA content. Upon analysis of outcome, only patients with either grade 2 or 3 atypia in patients without dysplasia on their index biopsy specimen showed progression to HGD/EAC, but the result of this analysis was not statistically significant, likely owing to type II statistical error. Overall, these results suggest that neoplastic progression in BE likely begins in the crypts at an early stage, before the onset of dysplasia, and that these changes may reflect a progressive accumulation of DNA abnormalities. Further studies using a larger cohort of BE patients with long-term outcome may help determine if the level of crypt atypia can serve as an important clinical biomarker of risk stratification in patients with BE.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Clinical Gastroenterology and Hepatology* at www.cghjournal.org, and at http://doi.org/10.1016/j.cgh.2023.10.007.

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Correspondence
Address correspondence to: Deepa T. Patil, MD, Department of Pathology, Harvard Medical School, 75 Francis Street, Boston, Massachusetts 02115. e-mail: dtpatil@bwh.harvard.edu.

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CRediT Authorship Contributions
Helen H. Wang (Data curation: Lead; Formal analysis: Lead; Methodology: Equal; Writing – original draft: Equal; Writing – review & editing: Equal; Co-First author: Equal)
Deepa T. Patil (Data curation: Equal; Formal analysis: Equal; Methodology: Equal; Writing – original draft: Equal; Writing – review & editing: Equal; Co-First author: Equal)
Thomas G. Paulson (Data curation: Equal; Formal analysis: Equal; Methodology: Lead; Writing – original draft: Equal; Writing – review & editing: Equal)
William M. Grady (Data curation: Equal; Formal analysis: Equal; Funding acquisition: Lead; Methodology: Equal; Supervision: Equal; Writing – original draft: Equal; Writing – review & editing: Equal)
Robert D. Odze (Conceptualization: Lead; Data curation: Equal; Formal analysis: Equal; Methodology: Equal; Supervision: Lead; Writing – original draft: Equal; Writing – review & editing: Equal)

Conflicts of interest
This author discloses the following: William M. Grady is a scientific advisory board member for Freenome, Guardant Health, and SEngine, a consultant for DiaCarta, Nephron, Guidepoint, and GLG, an investigator in a clinical trial sponsored by Janssen Pharmaceuticals, and receives research support from Tempus and LucidDx. The remaining authors disclose no conflicts.

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Data Availability
Data, analytic methods, and study materials will be made available to other researchers upon request.

Supplementary Methods

Endoscopy and Biopsy

All patients had endoscopies and biopsies performed with a large-channel endoscope and jumbo biopsy forceps using a turn-and-suction technique. After identification of endoscopic landmarks, systematic 4-quadrant endoscopic esophageal mucosal biopsy specimens were obtained every 1 to 2 cm during the baseline study endoscopy throughout the entire length of the BE segment. Multiple biopsy specimens also were taken of endoscopic abnormalities, when present. For patients who had 4-quadrant biopsy specimens obtained from every 1 cm of BE mucosa, every other level was analyzed such that the "1-cm protocol" was normalized to the "2-cm protocol." All biopsy specimens were fixed immediately in Hollande's solution and processed routinely for histologic evaluation. All 4-quadrant biopsy specimens were embedded into 1 paraffin block and serial 4-μm–thick tissue sections were cut and stained with H&E.

Data Transfer

All patients with complete clinical, histologic, flow cytometric, and follow-up data were deidentified with their names, dates of birth, addresses, and ethnicity removed from the data set, and were assigned a random identification number before their data were transferred to the authors' institutions.

DNA Content Flow Cytometry

Biopsy specimens were processed for flow cytometry by the single-step detergent method on an ICP-22 flow cytometer (Ortho Diagnostic Systems, Westwood, MA) and analyzed by the computer program Multicycle (Phoenix Flow Systems, San Diego, CA) as previously described.[1–3] Flow cytometric histograms were interpreted without knowledge of the histology results. An aneuploid population was defined as a G_0/G_1 population of cells that produced a discrete peak that was separate from the diploid population and constituted 2.5% or more of the cells in the biopsy specimen.[1] The percentage of cells in the 4N fraction was defined as a combined G_2/M-phase peak. The value greater than 6% was designated previously as increased and indicated an increased progression risk.[2]

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Supplementary Table 1. Criteria for Grading the Level of Atypia in the Basal Portions of the Crypts in Patients With Barrett's Esophagus

Grade	Histologic criteria
0	Architecturally normal or only mildly misaligned crypts without nuclear atypia, stratification, or mitoses, and with mature mucinous cytoplasmic differentiation either with or without goblet cells
1	Either normally aligned, or only slightly distorted and branched crypts, with normal or only mild nuclear enlargement, no or only mild hyperchromasia, no or only slight/focal loss of polarity and stratification that does not extend to the luminal surface of the basal crypts Mitoses may be present, but are few in number and not atypical Cytoplasm may show mild mucin depletion and goblet cells usually are present
2	Architecturally distorted crypts (irregular spacing, irregular shape, and branching) with moderate nuclear enlargement and hyperchromasia, at least focal areas of nuclear irregularity with variation in nuclear size and contour, focal mild nuclear stratification and loss of cell polarity that may reach the luminal surface of the basal crypts (but involving $<$50% of the circumference of the basal crypts), and often increased mitoses, some of which may be atypical Cytoplasmic mucin depletion is present Goblet cells may be dystrophic and maloriented along the crypt axis
3	Architecturally distorted crypts as in grade 2 (or worse), but with marked nuclear enlargement and elongation that is pencil shaped, irregularity, marked hyperchromasia, loss of cell polarity, and prominent stratification involving at least 50% of the circumference of the crypts (with extension to the luminal surface of the basal crypts) Mitoses may be abundant and focally atypical Cytoplasm shows little or no evidence of mucin differentiation Goblet cells often are dystrophic and maloriented