Evidence for DNA Damage Checkpoint Activation in Barrett Esophagus


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Abstract

Barrett esophagus is an epithelial metaplasia that predisposes to adenocarcinoma. Better markers of cancer risk are urgently needed to identify those patients who are likely to benefit most from emerging methods of endoscopic ablation. Disease progression is associated with genomic DNA changes (segmental gains, losses, or loss of heterozygosity). Although these changes are not easily assayed directly, we hypothesized that the underlying DNA damage should activate a DNA damage response (DDR), detectable by immunohistochemical (IHC) assays of checkpoint proteins and the resulting replicative phase cell cycle delays. Surgical specimens and endoscopic biopsies (N = 28) were subjected to IHC for the cell cycle markers cyclin A and phosphorylated histone H3 (P-H3), the DDR markers γH2AX and phosphorylated ATM/ATR substrates (P-ATM/ATRsub), and the DNA damage-responsive tumor suppressors p16 and p53. Correlations were made with histologic diagnoses. The fractions of cells that stained for cyclin A, P-H3, and γH2AX increased in parallel in dysplastic tissue, consistent with checkpoint-mediated cell cycle delays. Foci of nuclear γH2AX and P-ATM/ATRsub were demonstrated by standard and confocal immunofluorescence. Staining for p16 was more prevalent in early-stage disease with lower staining for γH2AX and P-H3. Staining for p53 was moderately increased in some early-stage disease and strongly increased in some advanced disease, consistent with checkpoint-mediated induction and mutational inactivation of p53, respectively. We suggest that IHC for DDR-associated markers may help stratify risk of disease progression in Barrett.

Translational Oncology (2010) 3, 33–42

Introduction

Barrett esophagus is characterized by asymptomatic replacement of normal squamous esophageal mucosa by intestinal metaplasia. Barrett is a major risk factor for esophageal adenocarcinoma (EAC), the only major cancer in the United States to be increasing in incidence. The population prevalence of Barrett is approximately 1% to 2%, with an estimated risk of progression to EAC of up to 0.5% per year [1,2]. The transformation of Barrett to EAC is typically marked by histologically...
defined steps of low-grade dysplasia (LGD) and high-grade dysplasia (HGD) [3]. However, the natural history of Barrett is unclear [4].

Most patients with documented Barrett participate in endoscopic biopsy surveillance programs with the goal of early identification of dysplastic change and the characterization of its severity. Although widely pursued, surveillance is of questionable utility [5]. Its benefit is limited by biopsy sampling error and perhaps more importantly, the reliability of histologic interpretation. Emerging evidence albeit with relatively short follow-up indicates that radiofrequency ablation can eradicate most dysplastic Barrett with acceptable morbidity [6]. However, successful therapy typically requires multiple treatment sessions for several months, and most of these patients would not have progressed to EAC. Improved markers are needed to direct endoscopic surveillance and ablation to those patients at high risk of EAC, to avoid unnecessary intervention and frequent surveillance in those at low risk, and to provide improved surrogate end points for ablation and chemoprevention trials.

Although the de facto criterion standard, histologic diagnosis is an imperfect determinant for clinical decision making [5,7,8]. As a result, many alternative biomarkers of increased risk have been proposed including telomere length [9,10], measures of apoptotic control [11], cell proliferation [12], loss of heterozygosity at several loci [13], and variation in nuclear DNA content [14,15]. Elegant studies have demonstrated substantial increase in cancer risk for those patients with aneuploidy, tetraploidy, or loss of heterozygosity of p53 and p16 [13,15,16]. However, the clinical utility of these molecular markers has been limited and none has become a standard component of clinical care today.

In the current study, we have examined Barrett tissue for evidence of a DNA damage response (DDR). Barrett tissue accumulates genomic changes with advancing dysplasia [9,17]. The highest risk tissue manifests p53 mutations, tetraploidy or aneuploidy, and widespread DNA copy number changes and loss of heterozygosity, indicative of chromosomal rearrangements, deletions, duplications, and/or gene conversion. Of particular note, recurrent lesions were seen at two DNA “fragile sites,” which are prone to strand breakage during DNA replication. Thus, the fragile site lesions and other genomic changes are typically caused by DNA double-strand breaks and/or other impediments to replication [18]. Such breaks are normally recognized by cells as a major threat to genome stability and cell viability and are met with a robust DDR.

We therefore hypothesized that DNA double-strand breaks and other impediments to DNA replication occur frequently in Barrett cells, are met with a DDR, and drive neoplastic progression. We sought evidence for a DDR in Barrett tissue, using molecular markers for the DDR and associated cell cycle phase delays and tumor suppressor gene activation. We emphasized methods that might be reproduced by most pathology laboratories and, thus, may prove to be clinically practical.

Materials and Methods

Tissue Acquisition

Banked Barrett tissues were used according to internal review board protocols at Fox Chase Cancer Center (FCCC), Cleveland Clinic, and Temple University. Surgical specimens were from FCCC, resected for HGD or EAC. The prospective portion of the study was performed at FCCC with separate internal review board approval. Informed consent was obtained from all patients. Patients were recruited who were scheduled for diagnostic endoscopy for newly diagnosed Barrett esophagus or surveillance endoscopy and endoscopic ablation for a known history of Barrett esophagus and/or associated intramucosal EAC. Additional biopsies were obtained beyond those taken for usual care. Samples were fixed in formalin and embedded in paraffin. Although collected from different institutions, the endoscopic samples were all read by one pathologist (H.C.).

Antibodies, Immunohistochemistry, and Immunofluorescence

Immunohistochemistry (IHC) was performed using the following antibodies (Abs): Cyclin A (NCL-cyclin A; Novocastra Laboratories Ltd, Newcastle upon Tyne, UK), P-H3 Ser10 (no. 9701; Cell Signaling Technology, Inc, Danvers, MA), P-(γ)H2AX Ser139 (Upstate 05-636; Millipore, Billerica, MA), P-ATM/ATR substrates (no. 2851; Cell Signaling Technology, Inc), p16 (JC2, a mouse monoclonal Ab that recognizes the first ankyrin repeat of p16; gift of Jim Koh, Duke University, Durham, NC), and p53 (NCL-p53-DO7; Novocastra Laboratories Ltd). IHC was performed by standard procedures using formalin-fixed, paraffin-embedded sections with antigen retrieval (microwave treatment in citric acid buffer). We used the avidin/biotin detection system with hematoxylin counterstain. Images were captured electronically [19], and cells with staining clearly above background were scored as positive. For validation of the IHC Abs using cultured cells, U2-OS osteogenic sarcoma cells were subjected to the designated treatments. Protein extracts and cells for IHC were prepared in parallel. Cells were embedded in Histogel (see below), fixed in formalin, embedded in paraffin, and sectioned as per the tissue samples.

Indirect immunofluorescence was performed by standard procedures using antigen retrieval by microwave treatment [20]. Secondary Abs used were Alexa Fluor 488 and 568 (Invitrogen, Carlsbad, CA). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images were obtained using a standard epifluorescence (Nikon, Melville, NY; TE300 inverted microscope with mercury bulb) and confocal microscopes (Nikon Eclipse 2000 TE inverted confocal microscope with C1si scanhead) with separate channels (laser lines 406 [DAPI], 488 [green], and 561 [red]).

Culture, Immunoblot Analysis, and Histologic Processing of U2-OS Cells

To validate the Abs used for immunohistochemistry, a U2-OS human osteogenic sarcoma cell line with inducible expression of p16 was cultured and subjected to immunoblot analysis as described [21], using the same Abs used for immunohistochemistry. Standard culture conditions included 1 μg/ml tetracycline to repress p16 expression. The following conditions were used to generate “low” and “high” levels of expression of each antigen. Cyclin A: low, treatment with 40 ng/ml nocodazole for 24 hours followed by two washes with phosphate-buffered saline and culture without drug for 4 hours; high, treatment with 4 μM hydroxyurea (HU) for 24 hours. P-H3: low, treatment with HU for 24 hours; high, nocodazole for 24 hours. γH2AX: low, standard culture; high, 50 μM etoposide for 24 hours, p16: low, standard culture conditions, with tetracycline; high, culture without tetracycline for 24 hours. p53: low, standard culture conditions; high, etoposide for 24 hours. Protein extracts were prepared in E1A lysis buffer. For immunohistochemistry, cells were scraped form the plate, pelleted by brief centrifugation, fixed in formalin for 5 minutes, solidified in a drop of Histogel (Thermo-Fisher, Waltham, MA), embedded in paraffin, and sectioned as per tissue. Immunohistochemical staining was performed as per the tissue samples.

Statistical Analysis

Proportions of cyclin A–positive and P-H3–positive cells in samples with or without dysplasia were compared with two-sample Wilcoxon rank
sum tests. Two-sided $P$ values were calculated for the null hypothesis of equal distributions and the alternative that the dysplastic distribution differed by a shift from the nondysplastic distribution. For comparison of $\gamma$H2AX staining in nondysplastic and dysplastic samples, the Jonckheere-Terpstra test was applied to the data presented in Table 1. The statistical software used was written at our institution by one of the authors (S.L.). Further details are available upon request.

**Results**

**Predicted Cell Cycle Phase Shifts**

A DDR imposes delays within replicative phases of the cell cycle—S and G2. These delays permit DNA repair before progression through mitosis fixes the mutations in progeny or mediates mitotic catastrophe [18,22]. Cyclin A is expressed in S and G2 phases and degraded in early mitosis. Given that early mitosis is short (<30 minutes) compared with S and G2 phases (8-12 hours), cyclin A expression can be used as a marker for the latter phases. Increased expression of cyclin A near the esophageal lumen has been correlated to degree of dysplasia in Barrett epithelium and taken as evidence for increased cell proliferation [12]. We hypothesized that a DDR contributes to the prevalence of cyclin A expression in Barrett.

Although the DDR delays mitotic entry, it might eventually increase the mitotic index. Such a scenario has been documented after a prolonged premitotic delay enforced by overexpression of the spindle checkpoint protein Mad2 [23]. Subsequent mitotic progression was inefficient, causing an increase in the mitotic fraction. This was thought to reflect decay of key mitotic factors during the prolonged G2 arrest. Thus, DDR checkpoint arrest may also result in mitotic delays and increased mitotic fractions. In addition, checkpoint responses typically mediate delays rather than enduring arrests. Cells with excessive damage may eventually slip into mitosis despite the presence of unrelicated or unrepaired DNA, resulting in defective sister chromatid separation ("anaphase bridges") and delayed mitosis [23,24]. Phosphorylation at Ser10 of histone H3 (P-H3) has been shown to tightly correlate with mitosis [25] and can be detected by IHC. We therefore reasoned that progression in Barrett might be associated with increased P-H3 staining.

For IHC, we chose Abs that have been established in the literature. In addition, we validated these reagents using cultured human cells synchronized in different cell cycle phases. In parallel, one culture was used to prepare protein extracts and assayed by immunoblot analysis, while the other was fixed, embedded, sectioned, and subjected to IHC with the same Ab. Low and high levels of cyclin A and P-H3 detected by immunoblot analysis were reflected in low and high levels of IHC staining, respectively (Figure 1, A–F).

**Surgical Resection Specimens**

We obtained Barrett tissues representing a broad spectrum of pathology, from simple Barrett to frank EAC. We began by examining surgical resection specimens from patients with HGD or EAC for IHC markers of replicative phases. Cyclin A expression was higher in Barrett tissue with dysplasia or EAC (Figure 2, C and E) than Barrett with no dysplasia (Figures 2A and 3A; $P < .04$), consistent with increased cell proliferation and/or replicative phase delays in dysplasia. Similarly, staining for P-H3 was minimal in nondysplastic Barrett (Figure 2B) but was readily detected in dysplasia or EAC (Figures 2, D and F, and 3A; $P < .006$). Thus, in this small series, dysplastic Barrett demonstrated an increase in cells within replicative phases, and P-H3 staining was a better discriminant for dysplasia than cyclin A staining.

**H2AX Formation**

The DDR is characterized by activation of the ataxia telangiectasia mutated (ATM) and ATM and Rad3-related (ATR) kinases [18]. ATM and ATR phosphorylate the variant histone H2AX on Ser129, generating its $\gamma$ form in chromatin surrounding DNA lesions [18,22]. $\gamma$H2AX formation is one of the most sensitive and specific markers of the DDR and is thought to foster a permissive local environment for recruitment and activation of other DDR proteins. To validate our $\gamma$H2AX IHC by immunoblot analysis, we prepared cell cultures with and without treatment with etoposide, a topoisomerase inhibitor known to induce double-strand DNA breaks. Etoposide treatment resulted in higher levels of $\gamma$H2AX by both assays (Figure 1, G–I).

We tested the surgical resection tissues for activation of the DDR by IHC for $\gamma$H2AX [26]. As expected, staining for $\gamma$H2AX in nuclei was not as uniform as that for cyclin A and P-H3, so it could not be as precisely quantified. Nonetheless, nuclear $\gamma$H2AX staining was readily detected in Barrett epithelium and was generally stronger in dysplasia or EAC than in nondysplastic Barrett tissue (Figure 2, G and H; data not shown).

**Endoscopic Biopsy Specimens**

We next tested whether these assays could be extended to endoscopic biopsies from patients undergoing routine screening or surveillance for dysplasia in Barrett. Such samples are used to stratify risk by pathological analysis and include tissues from patients who have not manifested HGD or EAC.

Cyclin A and P-H3 staining again correlated with dysplasia (Figure 3B, $P < .06$ and $P < .02$, respectively, Figure 4, A–D). In these analyses, samples diagnosed as indefinite for dysplasia were grouped with frankly dysplastic tissue, as is the norm, but the results remained significant when the indefinite samples were excluded ($P < .04$ and $P < .02$, respectively; data not shown). Again, P-H3 staining demonstrated a somewhat greater difference between nondysplastic and dysplastic groups than cyclin A staining (Figure 3B). $\gamma$H2AX staining was distinctly increased in nuclei in some samples (Figure 4, E and F; Table 1). A strong trend was observed toward increased $\gamma$H2AX staining in dysplastic samples, with only three of nine nondysplastic samples showing moderate $\gamma$H2AX staining versus six of seven dysplastic samples (Table 1; $P < .06$).

### Table 1. IHC of Molecular Markers in Barrett Endoscopic Biopsies by Dysplasia Grade.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Pathology</th>
<th>Cyclin A*</th>
<th>P-H3†</th>
<th>$\gamma$H2AX*</th>
<th>p16*</th>
<th>p53*</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>No dysplasia</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td>++</td>
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<tr>
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<td>+</td>
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<td>++</td>
<td>–</td>
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<td>–</td>
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<td>+</td>
<td>++</td>
<td>–</td>
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<td>No dysplasia</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>–</td>
</tr>
<tr>
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<td>++</td>
<td>++</td>
<td>–</td>
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<td>++</td>
<td>++</td>
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<td>–</td>
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<td>12</td>
<td>Indefinite for dysplasia</td>
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<td>++</td>
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<td>–</td>
</tr>
<tr>
<td>13</td>
<td>Indefinite for dysplasia</td>
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<td>+</td>
<td>++</td>
<td>+++</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>LGD</td>
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</tr>
<tr>
<td>15</td>
<td>HGD</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
<td>16</td>
<td>HGD, EAC</td>
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<td>+++</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
</tbody>
</table>

*N* indicates staining not available.

* * indicates staining in 1% to 5% of cells; ++, 6% to 20%; ++++, more than 20%.

† * indicates staining in 0.5% to 1.9% of cells; ++, 2% to 3%; ++++, more than 3%.
Phosphorylated ATM/ATR Substrates

We then assayed the DDR in Barrett tissue by a second method. ATM and ATR phosphorylate many additional substrates involved in the DDR. Among them are p53, p95/NBS1, MDM2, Chk2, BRCA1, CtIP, 4E-BP1, and Chk1 [22]. Peptide sequences with a hydrophobic residue followed by serine or threonine and then glutamine are preferred sites of ATM/ATR phosphorylation [27,28]. Antibodies directed against the phosphorylated forms of these peptides (P-ATM/ATRsub) have proven useful in detecting ATM/ATR–phosphorylated substrates, providing another marker of the DDR. We examined P-ATM/ATRsub reactivity by IHC. Barrett epithelial cells generally showed greater P-ATM/ATRsub than surrounding tissue, and a fraction of nuclei showed distinct nuclear staining (Figure 4, G and H).

p16 and p53 as DNA Damage-Responsive Tumor Suppressors

The two dominant tumor suppressors that are inactivated in Barrett—p16Ink4a and p53—can be induced by DNA damage, albeit with markedly different kinetics [29,30]. Thus, these proteins may be induced in Barrett by the DDR. p53 is the classic tumor suppressor induced by the DDR but can be induced by other stimuli [31–33]. Inserting so large external genotoxic insults, such as high-dose irradiation, p53 induction can contribute to a G1-S block. However, induction during replicative stress results in G2 arrest and, variably, apoptosis. Previous studies have documented p53 staining in some Barrett biopsies, although its relationship to a DDR has not been addressed [34–36]. Diffuse, high-level staining has been shown to correlate with p53 mutation in advanced disease (HGD or EAC) and to have some predictive power in LGD.

**Figure 1.** Validation of IHC staining. We exposed U2-OS cell clones with inducible expression of p16 to conditions predicted to yield low and high expression, respectively, of each target antigen (see Materials and Methods for additional details). Cells were processed in parallel for IHC (left, center) or immunoblot analysis (right (C, F, I, L, O)). The conditions were as follows. Cyclin A: low (A), synchrony in G1 using a mitotic arrest in nocodazole (Noc) followed by release for 4 hours; high (B), G1/S arrest mediated by hydroxyurea (HU). P-H3: low (D), G1/S arrest in HU; high (E), mitotic arrest in Noc. γH2AX: low (G), standard culture; high (H), induction of DNA double-strand breaks by etoposide. p16: low (J), standard culture (in tetracycline); high (K), withdrawal of tetracycline. p53: low (M), standard culture conditions; high (N), etoposide. Immunoblot analysis for tubulin (tub) served as a loading control. In immunoblots (I) and (O), intervening lanes (from an intermediate induction condition not shown) were removed digitally.
However, some p53 mutations in Barrett-associated neoplasia truncate the protein, abating its stable expression [34]. Therefore, p53 IHC alone cannot reliably detect advanced disease.

Although the regulation of p16 is incompletely understood, p16 is known to contribute to a cell cycle arrest mediated by the DDR [40]; we have found that p16 can be induced by DNA damage in a variety of primary human cells [30]. p16 is commonly inactivated during progression of Barrett esophagus [41–43], consistent with the notion that it restrains disease progression. Inactivation usually occurs by promoter methylation and is a relatively early event, preceding development of tetraploidy aneuploidy [17,44,45]. Limited IHC studies have shown p16 expression in some Barrett tissue [46,47] but have not examined its correlation with dysplasia or a DDR.

We assayed p16 expression by IHC, using conditions that we have extensively validated [20]. These conditions were further validated by induction of p16 in a cell line null for endogenous p16 expression (Figure 1, J–L). We observed a trend toward greater p16 detection in nondysplastic biopsy samples: five of nine samples with no dysplasia showed staining compared with two of seven dysplastic samples, with both p16-positive samples in the latter group scored as “indefinite” for

Figure 2. Increased cyclin A, P-H3, and γH2AX staining in Barrett-associated dysplasia within surgical resection specimens. Esophageal resection specimens from patients with HGD or EAC were subjected to IHC staining (brown) and counterstained with hematoxylin (blue). (A, B) Tissue with no dysplasia (ND). Cyclin A staining (A) is confined to nuclei of cells in basal regions of intestinal-like crypts. Note the goblet cells of Barrett. P-H3 staining (B) is rare. (C, D) LGD: Cyclin A and P-H3 staining are more prevalent. (E, F) EAC: Cyclin A and P-H3 staining are prevalent. (G) γH2AX staining is higher in EAC (left) than neighboring Barrett tissue without dysplasia (ND, right). (H) LGD: Scattered cells display distinct nuclear γH2AX staining.
dysplasia (Figure 4, I and J; Table 1). Only two of seven samples with p16 staining had moderate (++) γH2AX staining, and most had low (+) γH2AX staining. None had high (+++) P-H3 staining, suggesting that entry into mitosis may be restrained in p16-expressing tissue. Thus, a substantial fraction of nondysplastic or minimally dysplastic tissue detectably expressed p16. These tissues generally have evidence for only low-moderate DDR and none had high a mitotic index. We hypothesize that these represent lower-risk tissues, with a functional DDR.

We found that staining for p53 in Barrett biopsies increased with dysplastic progression (Figures 1, M–O, and 4, K and L; Table 1). Uniform high-level staining was seen in some samples with HGD or EAC, consistent with mutational inactivation of p53 that results in stabilization of the protein (Figure 5 C). Moderate p53 staining was observed in some samples with LGD, clearly before establishment of homogeneous neoplastic cell population (Figure 5 A). This latter pattern is consistent with functional p53 induction, potentially secondary to a DDR. Consistent with this notion, most (five of six) tissues with p53 staining had moderate γH2AX staining (Table 1). We hypothesize that these examples represent a strong DDR and stimulus for p53 induction. Note that some of these tissues either did not manifest dysplasia (Table 1, samples 3 and 4) or were indefinite for dysplasia (sample 11). We surmise that these tissues harbor increased risk for malignant progression, despite relatively benign pathology. Additional trends emerge when p16 and p53 staining results are combined. No samples (none of five) that showed p16 staining without p53 staining had greater than trace (+) γH2AX, whereas most (three of four) samples with p53 staining but no p16 staining did. Thus, use of p53 as part of a small panel of DDR-related markers may provide a more comprehensive picture of progression risk.

**Immunofluorescence for Nuclear DDR Foci**

As a further test for DDR in Barrett biopsies, we performed immunofluorescence for γH2AX and pATM/ATRsub. This staining would not be clinically practical but can detect these antigens in discrete nuclear foci. Such foci represent hallmarks of the DDR in cultured cells exposed to acute genotoxic insults [26,48]. Although background staining in the formalin-fixed tissue was greater than that typically seen in cultured cells, we observed individual Barrett cells with high nuclear γH2AX staining (data not shown), consistent with the IHC findings. Moreover, immunofluorescence staining demonstrated Barrett biopsies with distinct nuclear foci containing γH2AX and pATM/ATRsub (Figure 6, A–D). Confocal microscopy confirmed the nuclear location of the foci (Figure 6, E–H).

**Discussion**

We have presented evidence for a DDR in Barrett esophagus, characterized by staining for γH2AX and P-ATM/ATRsub, replicative phases of the cell cycle, and the DNA damage-responsive tumor suppressors p16 and p53. These markers were evident in surgical resection specimens,
endoscopic biopsies, and cytological brushings (data not shown). Some cells show a broad increase in nuclear staining for γH2AX and P-ATM/ATRsub. Others show distinct nuclear foci, confirmed by confocal microscopy. Staining for these markers is increased in dysplasia, consistent with evidence for development of widespread genomic DNA changes (deletions, gains, loss of heterozygosity) during disease progression in Barrett [17]. This DNA damage may be caused by replication under conditions of stress, such as sustained replication per se, exposure to genotoxic bile and acid [49], oncogene activation [50,51], reactive oxygen species [52], and telomere erosion [9]. We propose that DNA damage drives neoplastic progression and is countered by the DDR. We suggest that induction of p16 and p53, two major tumor suppressors that are inactivated in Barrett, is driven in part by the DDR and that disease progression is accelerated when these tumor suppressors are inactivated by promoter methylation (p16) or mutation (p16 and p53), relieving associated cell cycle inhibition and apoptosis.

Trends were observed for correlation between DDR and replicative phase markers. Increased cyclin A staining in surface metaplastic epithelium has been documented previously to correlate with advancing dysplasia in Barrett [12], where it was viewed as a marker of cell proliferation. We suggest that increased cyclin A staining may also reflect S and G2 delays mediated by the DDR. We have found examples of tissue without dysplasia in which the fraction of cells that stain for cyclin A is as high or higher than in most cases of HGD or EAC but P-H3 staining is low and p53 staining is observed (Table 1, samples 3 and 4). This finding may reflect the operation of an intact DDR, with functional delays in S and G2 progression mediated by cyclin-dependent kinase inhibition. With increasing DNA damage and loss of functional p16, p53, and perhaps other DDR effectors, unrestrained S and G2 progression may result in a lower fraction of cells in those cell cycle phases and an increased mitotic fraction. Consistent with this notion, P-H3 staining was high in most cases of HGD and EAC (cf., Table 1). Increased P-H3 staining in fact showed a modestly better correlation with dysplasia than cyclin A staining (Figures 2–4).

Although we have established a correlation between DDR and associated cell cycle markers on the one hand and pathological diagnosis on the other, the latter is widely recognized as an imperfect, default criterion standard. A key question now is whether these molecular markers can improve prediction of EAC risk beyond that provided by histologic diagnosis alone. A larger prospective study will be required to address this issue. However, we note that, in the examples shown in Figure 3, absence of p16 staining and increased γH2AX, P-ATM/ATRsub, and p53 staining distinguished the Barrett sample without dysplasia but with a history of HGD from the sample with simple Barrett (no history of advanced disease). Thus, a small panel of DDR markers may identify tissue at increased risk, despite absence of dysplastic morphology.

**Figure 4.** Increased staining for replicative cell cycle phases, DDR, p16, and p53 in Barrett endoscopic biopsy samples. Nearby formalin-fixed sections were stained from samples with no dysplasia and no history of HGD or EAC (left) or no dysplasia but with a history of HGD (right). Sections were stained for (A, B) cyclin A, (C, D) P-H3, (E, F) γH2AX, (G, H) pATM/ATRsub, (I, J) p16, and (K, L) p53. p16 staining was more prominent in the specimen with Barrett alone (I), whereas pATM/ATRsub (H) and p53 (L) staining were more prominent in the sample with a history of HGD.

**Figure 5.** Two patterns of p53 staining in Barrett tissue. Endoscopic biopsies were subjected to IHC for p53. (A) LGD showing moderate p53 staining in nuclei of scattered cells. Note the diversity of cell types in the epithelium, indicating that this tissue is not neoplastic. (B) No p53 staining in a sample with no dysplasia (ND). (C) Uniformly high p53 staining in a sample with uniform cell morphology indicative of HGD or EAC.
A definitive test of this notion will require a larger sample set and longitudinal follow-up data.

In summary, we have presented evidence for a DDR in Barrett esophagus based on staining for γH2AX and P-H3, elevated fractions of cells in replicative phases, and expression of DDR-associated tumor suppressors. We observed increased staining for cyclin A and P-H3 in dysplastic tissue, consistent with the notion that DNA damage and the DDR impose replicative phase delays. We observed trends toward p16 staining in tissues with little or no dysplasia and p53 staining in tissues with increasing dysplasia. Finally, examples were found of tissue without dysplasia that manifested high cyclin A, P-H3, γH2AX, and/or p53 staining. We suggest that these markers might add value to pathological diagnosis in identifying high-risk tissue and merit testing in a larger set of tissues and in patients with longitudinal follow-up.

Acknowledgments
The authors thank Michal Jarnik for assistance with confocal imaging, Michael Slifker for assistance with the statistical analysis, and Oleh Haluszka, M.D., Minh Nguyen, M.D., JoEllen Weaver, and Joanne Magen (FCCC) for prospective collection of patient samples.

DNA Damage Response in Barrett von Holzen et al.


