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Agrin loss in Barrett's esophagus-related neoplasia and its utility as a diagnostic and predictive biomarker

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Abstract

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The authors declare that they have no competing financial interests.

Purpose: There is an unmet need for identifying novel biomarkers in Barrett's esophagus (BE) that could stratify patients with regards to neoplastic progression. We investigate the expression patterns of extracellular matrix (ECM) molecules in BE and BE-related neoplasia, and assess their value as biomarkers for the diagnosis of BE-related neoplasia and to predict neoplastic progression.

Experimental Design: Gene expression analyses of ECM matrisome gene sets were performed using publicly available data on human BE, BE-related dysplasia, esophageal adenocarcinoma (ADCA) and normal esophagus. Immunohistochemical expression of basement membrane (BM) marker agrin (AGRN) and p53 was analyzed in biopsies of BE-related neoplasia from 321 patients in three independent cohorts.

Results: Differential gene expression analysis revealed significant enrichment of ECM matrisome gene sets in dysplastic BE and ADCA compared with controls. Loss of BM AGRN expression was observed in both BE-related dysplasia and ADCA. The mean AGRN loss in BE glands was significantly higher in BE-related dysplasia and ADCA compared to non-dysplastic BE (NDBE; $p<0.001$; specificity=82.2% and sensitivity=96.4%). Loss of AGRN was significantly higher in NDBE samples from progressors compared to non-progressors ($p<0.001$) and identified patients who progressed to advanced neoplasia with a specificity of 80.2% and sensitivity of 54.8%. Moreover, the combination of AGRN loss and abnormal p53 staining identified progression to BE-related advanced neoplasia with a specificity and sensitivity of 86.5% and 58.7%.

Conclusions: We highlight ECM changes during BE progression to neoplasia. BM AGRN loss is a novel diagnostic biomarker that can identify NDBE patients at increased risk of developing advanced neoplasia.

Keywords

Agrin; Barrett's esophagus; Biomarker; Extracellular matrix; Immunohistochemistry

Introduction

Incidence of esophageal adenocarcinoma (ADCA) has rapidly increased in the United States and most Western countries during the last few decades (1-4). The strongest risk factor for ADCA is the presence of Barrett's esophagus (BE), a premalignant condition defined by replacement of normal squamous epithelium of the esophagus by intestinal-type metaplastic columnar epithelium (5-9). Neoplastic transformation from BE to ADCA occurs through a sequence of genetic and epigenetic alterations associated with histopathologic changes, in which Barrett's epithelium without dysplasia (non-dysplastic BE, NDBE) evolves to BE with dysplasia, eventually culminating in ADCA (10,11).

To reduce cancer risk, current guidelines recommend endoscopic surveillance in BE patients (12). The BE segment is randomly sampled, but in practice sampling variability limits the ability to reliably detect neoplasia (13) The identification of dysplasia remains the standard of care for risk stratification, although this approach also has limitations, most significantly the poor interobserver agreement, particularly at the lower and higher end of the neoplastic spectrum (14-16).

There is currently an unmet need for diagnostic and predictive biomarkers in BE-related neoplasia. Several immunohistochemical (IHC) potential biomarkers have been studied in BE progression (17-19). Although, p53 stain shows significant promise, so far, neither this stain nor any other marker has been implemented in routine practice (20-24).

Although the increased expression of extracellular matrix (ECM) proteins plays a crucial role in inflamed, fibrotic and neoplastic tissues (25-27), its role in BE progression is poorly understood. ECM genes shown to be upregulated in ADCA when compared to BE and BE-related dysplasia include tenascin-C and fibronectin, several metalloelastases and their inhibitors, laminins, heparanase and others (28-32). To date, however, no comprehensive data on the ECM matrisome (i.e., the ensemble of collagens, glycoproteins, proteoglycans, basement membrane [BM] and associated proteins) (33) in ADCA or its precursor lesions in BE patients are available.

To better understand changes of the ECM in BE-related neoplasia, we evaluated the presence of ECM molecules using gene expression analysis of two publicly available datasets and performed IHC on human biopsies to evaluate potential ECM biomarkers to identify patients who are at higher risk of neoplastic progression. Our studies highlight significant changes in the ECM during progression of BE to neoplasia. We identify the utility of BM marker AGRN; **1)** as a potential novel diagnostic biomarker to identify BE-related neoplasia with a specificity of 82.2% and sensitivity of 96.4%, and **2)** that AGRN loss in combination with abnormal p53 IHC can identify progression to advanced neoplasia with a specificity of 86.5% and sensitivity of 58.7%.

Materials and Methods

Patient samples and histopathologic review

In total, we analyzed 481 formalin-fixed, paraffin-embedded (FFPE) biopsies of Barrett's esophagus (BE) and BE-related neoplasia from 321 patients undergoing endoscopy between 1991 and 2019. For histopathological analysis, slides were stained with hematoxylin and eosin (H&E) and reviewed by five gastrointestinal pathologists (GI) with expertise in BE pathology: cohort #1 (VD, ARM, LZ) and cohorts #2 and #3 (VD, RDO, DTP) (see below for cohort details). Pathologists were blinded to the original diagnosis. All cases were categorized as normal esophagus, NDBE, BE with low-grade dysplasia (BE-LGD), BE with high-grade dysplasia (BE-HGD) and esophageal adenocarcinoma (ADCA). The final diagnosis was based on consensus or majority (2 of 3) opinion with agreement between two or more pathologists. All non-consensus or BE indefinite for dysplasia samples (n=20) were excluded from the study. Figure 1 summarizes the details of the study and individual samples investigated for each cohort.

We evaluated three distinct cohorts of patients with BE (Fig. 1): **cohort #1)** Our initial sample set including non-dysplastic and neoplastic BE biopsies to examine the loss of AGRN in the BM of BE glands during BE progression; **cohort #2)** cases with long term follow-up to examine specifically the ability of AGRN loss to identify progression to BE-HGD or ADCA compared to a control cohort that did not develop neoplasia; and **cohort #3)** a multi-institution group to confirm our findings in cohort #2.

Cohort #1: Barrett's esophagus and Barrett's esophagus-related neoplasia samples—This initial study cohort (Massachusetts General Hospital, MGH) included samples from 129 patients with the following diagnoses: NDBE (n=73), BE-LGD (n=22), BE-HGD (n=26) and ADCA (n=8) (Fig. 1, Table 1). Clinicopathological parameters including age at diagnosis, gender and length of BE segment (see Table 1 for details) were recorded.

Cohort #2: Non-progressors and progressors samples—This cohort (MGH) included samples from 161 patients that were divided into 2 groups, progressors and non-progressors (Fig. 1, Table 2). A progressor was defined as a patient who developed advanced neoplasia (BE-HGD/ADCA) after at least one year following the initial diagnosis of BE. A non-progressor was defined as a patient who did not develop dysplasia or ADCA during a long-term surveillance period (mean duration between the 1st and 2nd biopsy is 9.4 years). In addition to documenting clinicopathological parameters such as age at diagnosis, gender, body mass index, history of smoking, history of alcohol consumption and length of BE segment, we also recorded the time interval (in years) between NDBE biopsies and subsequent index biopsy (see details below). Index biopsy from progressors was defined as the first biopsy with dysplasia and that for non-progressors was defined as the most recent non-neoplastic biopsy (see Table 2 for details). All the biopsies were reviewed by three gastrointestinal pathologists (VD, RDO, DTP).

1) Progressor samples: We interrogated the files of one institution and identified FFPE material from at least one NDBE biopsy, prior to the diagnosis of advanced neoplasia (BE-HGD/ADCA). The progressor group consisted of 93 NDBE biopsies. In 54/93 cases we examined a single NDBE biopsy, in 32/93 cases we evaluated two NDBE biopsies and in 7/93 cases we examined more than 3 serial biopsies per patient obtained at different time points prior to the index neoplastic sample. In addition to NDBE biopsies, the index dysplastic biopsies with BE-HGD/ADCA from 65 patients were also analyzed. The mean duration between last pre-dysplastic biopsy and index dysplastic biopsy was 3.9 years (range: 1 to 18 years).

2) Non-progressor samples: For the non-progressor group, we identified NDBE biopsies from 96 patients who did not develop neoplasia upon follow-up endoscopies. We examined a single NDBE biopsy for all 96 patients and additionally for 32/96 patients we also evaluated a second NDBE biopsies obtained at different time points prior to the most recent non-dysplastic sample. The mean interval between the index and the most recent non-dysplastic biopsy was 9.4 years (range: 2 to 20 years).

Cohort #3: Multi-institutional evaluation samples—Our third cohort included 62 samples from 31 BE progressors collected at multiple institutions [Boston University (n=7), Brown University (n=17), and University of California San Francisco (n=7)] and was used to confirm our observations from cohort #2. We evaluated the NDBE biopsy (n=31) as well as 10 BE-LGD, 16 BE-HGD and 5 ADCA samples.

Rat model

A modified Levrat model with end-to-side esophagojejunostomy to induce gastroduodenoesophageal reflux to develop BE-related dysplasia and ADCA was investigated in this study and sample collection methods were previously described in detail (34).

Antibodies

Primary antibodies used for immunohistochemistry (IHC) were: rabbit anti-AGRN (1:250; #NBP1-90209, Novus Biologicals, Littleton, CO), rabbit anti-Collagen IV (1:5000; #ab6586, Abcam, Cambridge, MA), goat anti-Collagen XVIIIA1/Endostatin (1:250; #AF1098, R&D Systems, Minneapolis, MN), rabbit anti-Heparan Sulfate Proteoglycan 2/HSPG2 (1:750; #PB9277, Boster, Pleasanton, CA) and mouse anti-p53 (1:200; #M700129-2, Clone DO-7, Agilent, Santa Clara, CA).

Immunohistochemistry

IHC stains were performed on the following samples: Progressor cohort [1] one or more BE-ND biopsies prior to the diagnosis of dysplasia, and [2] the index biopsy with dysplasia; Non-progressor cohort; [1] index biopsy at diagnosis of BE, and [2] the last available surveillance BE biopsy. IHC was performed as recently described using Thermo LabVision Autostainer 360 automated staining system (35). Briefly, individual sections were dewaxed, rehydrated and either stained with H&E following standard procedures using the Shandon Varistain Gemini ES Automated Slide Stainer (Thermo Fisher Scientific) or treated with heat-induced epitope-retrieval (HIER) prior to immunostaining. The sections were incubated in 10mM sodium-citrate (pH6.0) or 10mM Tris (pH9.0) buffered solutions containing 0.05% Tween at 125°C for 5min using a decloaking chamber (Biocare Medical, Concord, CA). For optimal AGRN IHC, enzyme digestion using 0.2% pepsin in 0.2N HCl (Agilent, Santa Clara, CA) according to the manufacturers' protocol was done prior to the immunostaining. Sections were subsequently pretreated using BLOXALL endogenous enzyme blocking solution (Vector Laboratories, Burlingame, CA) for 10min. After blocking with normal horse serum, the sections were incubated with individual primary antibodies for 1h followed by secondary ImmPRESS polymer detection systems (Vector Laboratories) according to the manufacturer's protocol. Subsequently, the Vulcan Fast Red Chromogen Kit 2 (red staining; Biocare Medical) was applied as substrate. Hematoxylin was used as final counterstain. Image documentation was done using the Leica Aperio AT2 slide scanner system (Leica Biosystems Imaging, Nussloch, Germany). Appropriate positive and negative controls for IHC were performed for each set of slides.

Histological and immunohistochemistry scoring

Assessment of AGRN loss—To ensure an unbiased review, AGRN immunostains were blindly reviewed by one investigator (SR) in conjunction with a single gastrointestinal pathologist (VD), both blinded to the diagnosis (both original and consensus) and clinical data. All H&E and AGRN stained slides used for scoring were scanned at 20X and digitized using a Leica Aperio AT2 slide scanner. The Aperio ImageScope Annotations Tool was used to circle the individual areas of interest.

We examined the percentage loss of AGRN stain in **1)** all BE glands on the section and **2)** only neoplastic region as assessed by one gastrointestinal pathologist (VD). The area with loss of basement membrane (BM) AGRN was calculated and expressed as 1) area with loss of BM AGRN/total area of BE glands and 2) area with loss of BM AGRN/area of neoplastic BE glands. Individual regions were summed and used as area of AGRN loss within a sample. In order to confirm loss of AGRN expression, we required the adjacent glands or blood vessels to show positive BM stain (internal positive control). Normal esophageal squamous mucosa, muscularis mucosae, and any sampled submucosa were not considered in this study. In addition to assessing percentage loss of AGRN staining within the region of interest, we also analyzed loss of expression by number of glands in a subset of progressor cases.

Automated quantitation of P53 staining

P53 IHC was performed on consecutive sections of all biopsies evaluated for AGRN loss from cohort #2. All slides were scanned using a Leica Aperio AT2 slide scanner system, and staining was analyzed using VIS (Visiopharm) histopathology image analysis software. Nuclear staining of p53 was graded as: 1+ (weak), 2+ (medium) and 3+ (strong) p53-positive cells on the basis of staining intensity. For quantification, the BE glands were hand-annotated for analysis by one pathologist (AN) and p53 intensity was assessed using the VIS app 10002 ER, modified for p53 stain. For this study, an abnormal p53 stain [defined as 3+ staining or loss of staining (null cell phenotype)] was used for comparative analysis.

Ethics

The study was approved by the Partners Institutional Review Board protocol (IRB 2018P185) and the MIT IRB (#1408006568). The study was conducted in accordance with the U.S. Common Rule and performed on discarded human tissue samples and is thus exempt from direct patient consent.

Gene expression data and analysis

We analyzed two different types of gene-expression data sets; [**1**] genome-wide gene expression microarray data (36) and [2] whole transcriptome RNA sequencing data (37) (Supplementary Table S1-S3).

Maag et al. (2017) (37) data were downloaded from Array Express (E-MTAB-4054). DESeq2 (v1.24.0) running under R 3.6.0 was used for differential expression analysis using count data as input. Variance-stabilized transformation was used to export normalized log2 expression values for gene-expression visualization with Tibco Spotfire Analyst version 7.11.1. Pre-ranked Gene Set Enrichment Analysis (v4.0.3) was run using DESeq2 Wald statistic as a ranking metric and the canonical pathways gene set collection (~2200 gene sets) from MsigDB (v7.0). Supplementary Table S2 contains the matrisome category annotations, normalized expression scores (NES) for protein coding genes, Wald statistic ranking metrics for the comparisons used in this study and group averages.

Kim et al. (2010) (36) data were downloaded from the Gene Expression Omnibus (GSE13898). Standard Gene Set Enrichment Analysis (v4.0.3) was run using downloaded log2 expression values and the canonical pathways gene set collection from MsigDB (v7.0). Supplementary Table S3 contains the matrisome category annotations, normalized expression values and group averages.

Statistical analysis

All statistical analyses were performed using SPSS statistical software (version 20.0) and Graphpad Prism (version 7). All categorical clinicopathological parameters were analyzed using Chi squared or Fisher's Exact test, Student paired or unpaired two-tailed t test and all continuous variables are analyzed using Mann Whitney U test. A p-value <0.05 was considered to be statistically significant. Receiver Operating Characteristics (ROC) curve analysis was performed to generate the optimum cut-off point for continuous variables. ROC curve was created by plotting the true positive rate (sensitivity) against the false-positive rate (1–specificity) at various threshold settings. The area under the ROC curve (AUC) is a measure of how well a parameter can distinguish between two diagnostic groups (diseased/normal).

Results

Validation of marked extracellular matrix changes during esophageal carcinogenesis

To identify novel biomarkers which can assist the diagnosis or progression of BE-related neoplasia, we investigated the expression of ECM molecules. First, we analyzed the mRNA expression levels of ECM (matrisome) gene sets against the lists of expressed genes in two publicly available datasets (Figs. 2, S1-S3, Supplementary Tables S1-3) (36,37).

The gene-expression analysis was conducted by comparing previously defined lists of genes (gene sets) representing [1] the entire set of ECM-related genes (matrisome) plus [2] multiple defined subsets of that entire list, such as collagens, proteoglycans, glycoproteins, basement membranes (BM), ECM regulators, ECM-associated growth factors, as previously defined (33), against the lists of genes altered during BE progression as measured by microarray Kim et al. 2010) (36) or RNA-seq (Maag et al. 2017) (37). As controls we used a database (MSigDB) of 2200 gene sets unrelated to ECM, curated by the Broad Institute). We analyzed data for 63 samples (37) including NDBE (n=19), BE-LGD (n=8), ADCA (n=17) and normal esophageal squamous samples (N; n=19) as well as from 118 samples (36) including NDBE (n=2), BE-LGD (n=7), BE-HGD (n=6), ADCA (n=75) and non-tumor esophageal tissues (n=28), respectively.

Using this approach, we found enrichment of the entire matrisome gene set and of many of the individual matrisome subsets among the most upregulated genes in NDBE and neoplastic BE stages (BE-LGD / BE-HGD or ADCA) as compared to control esophageal tissues (Figs. 2A, 2B, S1A and S1B). In both datasets, however, we noticed in particular the significant enrichment for the gene sets of the core matrisome category (structural proteins such as collagens, proteoglycans and glycoproteins, and BM proteins) whereas the matrisome-associated gene sets (ECM regulators, secreted factors) were less markedly

enriched (Figs. 2B, S1B). Given this consistent upregulation, we next performed gene set enrichment analysis (GSEA) of the core matrisome and its three sub-categories; collagens, proteoglycans and the BM gene sets. We identified the most significant enrichment of these four gene sets in ADCA relative to normal controls (ADCAvN; Figs. 2C, S1C), with enrichment scores ranging between 1.97 and 2.25 for Maag et al. (37) (Fig. 2C) or 2.28 and 2.93 for Kim et al. (36) (Fig. S1C), respectively. In addition, using GSEA for these 4 gene sets, we also identified their significant enrichment in early stages of BE progression, comparing NDBE relative to normal (N) controls (NDBEvN) as well as BE-LGD relative to normal controls (BE-LGDvN) for both datasets (Supplementary Fig. S2).

Although both the core matrisome and the BM gene sets were found to be highly enriched in all comparisons, and since the BM category represents a feasibly sized set of genes and showed better enrichment compared to the complete collagen and proteoglycan gene sets in many comparisons, we selected the BM gene set and evaluated the individual genes in this category more closely. Heatmaps of RNAseq expression values (37) (Supplementary Fig. S3A) or array hybridization signals (36) (Supplementary Fig. S3B) for the individual genes in the BM matrisome category clearly show that many genes are highly upregulated in the majority of samples during BE progression and in ADCA when compared to normal esophageal samples (Supplementary Fig. S3 and Supplementary Tables S2 and S3).

AGRN basement membrane loss during BE development

Given that ECM genes are upregulated during progression through NDBE to neoplastic states, we performed extensive IHC screening on FFPE samples to evaluate the potential diagnostic value of relevant ECM proteins (Figs. 3, S4-S8). To investigate the diagnostic utility of BM proteins as potential biomarkers, we selected previously validated antibodies (35) specific for agrin (AGRN), heparan sulfate proteoglycan 2 (HSPG2), collagen IV (COLIV) and collagen XVIII alpha 1 (COLXVIII A1) and performed IHC on consecutive sections of NDBE, BE-LGD, BE-HGD and ADCA (Supplementary Fig. S4). In accordance with the gene expression data (Supplementary Fig. S3), we detected increased staining intensity for COLIV, COLXVIIIA1 and HSPG2 in BE-related dysplasia and ADCA samples compared to normal samples. BM staining was consistently present in normal esophageal squamous mucosa, NDBE, BE-LGD and BE-HGD, although partially lost in ADCA samples. However, in addition to BM staining, these stains also showed diffuse reactivity throughout the connective tissue.

In contrast, we noticed strong and selective staining for AGRN in the BM of blood vessels, normal squamous epithelium and NDBE glands, while the BM reactivity in BE-LGD, BE-HGD and ADCA was frequently lost, either focally or diffusely (Supplementary Fig. S4), a finding that we previously observed in the progression of sessile serrated lesions to dysplasia (38). Given the lack of a consistent and clear differential immunoreactivity with HSPG2, COLIV and COLXVIII A1 in normal versus BE and ADCA tissues, and the optimal differential staining observed between normal and abnormal glands, we elected to focus further on evaluation of AGRN.

To examine the loss of AGRN in the BM of BE glands during BE progression in more detail, we stained parallel sections from esophageal squamous mucosa, NDBE, BE-LGD,

BE-HGD, and ADCA for AGRN and compared it to the well-known BM protein COLIV (Fig. 3). We noted loss of AGRN in biopsies from BE-LGD, BE-HGD, and ADCA (Fig. 3). In contrast, COLIV was consistently present in all biopsies with the exception of ADCA. To validate this consistent loss of BM AGRN reactivity in BE-related neoplasia, we stained additional samples from a variety of patients and confirmed these results (Supplementary Fig. S5), representative positive and negative controls for AGRN are shown respectively (Supplementary Fig. S6).

To further evaluate the biological relevance of these findings, we assessed the BM AGRN loss during esophageal disease progression in a Levrat model of BE carcinogenesis (34) (Supplementary Fig. S7). In concordance with the human samples, AGRN immunostaining confirmed the consistent presence of AGRN in the BM of normal esophageal squamous epithelium and NDBE glands, and loss of this protein in the BM of BE-LGD, BE-HGD and ADCA (Supplementary Fig. S7). In contrast, COLIV is consistently present in all samples with the exception of ADCA. Collectively, the Levrat model of BE carcinogenesis recapitulates the patterns of AGRN loss seen during human progression of BE.

Quantitative analysis of AGRN in normal esophagus, BE and BE-related neoplasia (cohort #1)

In total, we analyzed 481 FFPE biopsies of BE and BE-related neoplasia from 321 patients ranging from 26 to >90 years of age undergoing endoscopy between 1991 and 2019 as discussed below. A quantitative assessment of AGRN was performed (as described in Materials and Methods and Supplementary Fig. S8) in biopsies from 129 patients of BE and BE-related neoplasia (cohort #1, for study overview see Fig. 1), representing the following pathologic diagnoses that were validated by 3 observers: NDBE (n=73), BE-LGD (n=22), BE-HGD (n=26), ADCA (n=8) and normal esophageal squamous epithelium (n=64).

Table 1 shows the clinicopathologic features of the primary cohort#1 and summarizes the AGRN loss in the BM of glands in BE and BE-related neoplasia samples. In patient samples with NDBE, the mean percentage of AGRN BM loss was 0.9% (range 0-5.8%, no discernable loss n=37/73). In BE-LGD, the mean percentage AGRN loss was 19.1% (range 3.3-45.8%) within the dysplastic region, whereas in BE-HGD mean percentage AGRN loss was 16.1% (range 0-66.6%). Finally, in ADCA, the mean percentage AGRN loss was 45.8% with a loss ranging from 14.1-78.4% in the neoplastic regions of individual samples. The mean AGRN loss was significantly higher in BE-related neoplasia (combining BE-LGD/BE-HGD/ADCA) compared to NDBE ($p<0.001$). Also, of the 129 biopsies stained for AGRN for cohort #1, we found AGRN staining present in the BM of the squamous mucosa in all 64 samples that included normal esophageal squamous mucosa.

Based on receiver operating characteristic (ROC) analysis of AGRN loss (Fig. 4), NDBE cases could be distinguished from neoplasia (BE-LGD/BE-HGD/ADCA) at a cut-off ≥2% with a specificity and sensitivity of 82.2% and 96.4%, respectively, showing an area under curve of 0.951 (Fig. 4). At a cut-off ≥5% AGRN BM loss, the specificity was higher (95.9%) albeit with a lower sensitivity of 87.5%. Taken together, this finding of AGRN BM loss in BE-related dysplasia/neoplasia compared to BE-NDNDBE supports the potential diagnostic utility of AGRN loss as a novel biomarker for progression of BE-related neoplasia.

Comparison of clinical features between BE-progressor and Non-progressor samples (cohort #2)

We next evaluated an additional larger sample set (cohort #2, Fig. 1) of 161 patients to evaluate AGRN loss as a potential predictive marker of progression to advanced neoplasia (BE-HGD/ADCA). This cohort #2 is comprised of 2 groups of patients. **1)** The progressor group consisted of 65 patients from whom 93 NDBE biopsies, prior to the diagnosis of neoplasia, were analyzed and compared with 65 index biopsies with BE-HGD/ADCA and **2)** The non-progressor group included 132 NDBE biopsies (1st biopsy n=96, 2nd biopsy n=36) from 96 patients who did not develop neoplasia over a long follow-up (mean 10 years, range: 2 to 20 years).

Compared to the non-progressor group (Table 2), the progressor group showed a greater proportion of men (86.2% vs 64.6%, p=0.002). Long BE segment ≥ 3 cm was significantly associated with progressors compared to non-progressors (75.4 vs 34.1%, p<0.001). However, there was no significant difference in body-mass index, smoking or alcohol intake between the two groups. Of the 65 BE-progressor patients, 30/65 (46.2%) progressed to BE-HGD and 35/65 (53.8%) to ADCA (Table 2).

Table 3 summarizes the evaluation of percent loss of AGRN-positive area for the individual samples, showing comparison of the initial diagnosis (Table 3A, n=272) and the consensus diagnosis (Table 3B, n=242) for cohort #2. We predominantly noted AGRN loss in advanced neoplasia biopsies in the progressor group (Table 3).

AGRN loss in non-dysplastic BE biopsies from non-progressor and progressor groups

There was a significant difference in the percentage of AGRN loss in the BM of BE glands between NDBE biopsies from the progressor group compared to the non-progressor group (2.1% vs 0.6%; p<0.001; Supplementary Table S4). In order to distinguish progressor NDBE from non-progressor NDBE, we next explored the optimal cut-off points on a ROC curve for the AGRN loss and found that most non-progressor NDBE biopsies (80.2%) had <1% loss of AGRN compared to 45.2% in the progressor NDBE samples (Supplementary Table S4). When a cut-off of ≥1% loss was used, 54.8% of progressor biopsies demonstrated AGRN loss compared to 19.8% of non-progressor biopsies, and this difference was statistically significant (p<0.001). ROC analysis revealed that using a ≥1% cut-off, the sensitivity and specificity for separating progressors from non-progressors based on NDBE samples was 80.2% and 54.8%, respectively (Supplementary Fig. S9). At a cut-off ≥2% AGRN BM loss, the specificity was higher (92.7%) but sensitivity dropped to 38.7%. Multivariate logistic regression analysis was performed to see the independent significance of AGRN and to remove the effect of the confounders (Supplementary Table S5).

In addition to assessing percentage AGRN loss by selecting the area of interest, we also analyzed loss of AGRN expression by number of glands affected to determine whether the percentage thresholds could be translated to thresholds that could be used on a more practical basis (Supplementary Table S6). We performed this sub-analysis in 47 NDBE samples from the progressor cohort and found that the average number of BE glands showing loss of expression corresponding to the percentage thresholds described above was

as follows: 0% AGRN loss: no glands, 0.1-1% AGRN loss: 4 glands; 1.1-2%: 5 glands; >2%: 17 glands (Supplementary Table S6). Thus, the 2% agrin loss would correspond to loss in 5 glands.

Comparison of AGRN loss in patients with multiple samples of cohort #2

AGRN loss of ≥1% in the BM of BE glands was observed in all biopsies harboring BE-HGD/ADCA, supporting the high sensitivity of AGRN loss for the diagnosis of advanced neoplasia (Supplementary Table S7A). Among the 65 patients in the progressor group, we identified 24 who had two sets of NDBE biopsies, prior to the diagnosis of neoplasia, available for analysis. The first biopsy was the sample used to establish the diagnosis of BE while the second biopsy was obtained proximate in time to the index biopsy with advanced neoplasia. Biopsies more proximate to the index biopsy with advanced neoplasia showed a higher AGRN loss compared to the first biopsy, but this difference was not statistically significant (Supplementary Table S7B), suggesting the loss of AGRN BM expression as an early event in NDBE biopsies of progressors.

Within the non-progressor cohort, we compared the index BE biopsy which established a diagnosis of BE and the last available surveillance biopsy in 36 patients from the non-progressor group (Supplementary Table S7C) and found that the majority of samples showed only low AGRN loss (<1%), representing 80.6% of the first biopsy and 75% of second biopsy, respectively. The comparable results of AGRN loss between the first biopsy and last available surveillance biopsy highlights the consistent low percentage of AGRN loss in NDBE biopsies of non-progressors over time.

Utility of p53 and AGRN immunohistochemistry in combination to identify the progression to BE-related advanced neoplasia

Given the current literature that aberrant p53 expression is associated with an increased risk of developing BE-HGD or ADCA (see also Introduction), we next evaluated p53 expression on sections consecutive to those used for AGRN IHC for cohort #2 (Fig. 5). ROC analysis of abnormal p53-positive cells in NDBE progressor samples compared to ND-BE non-progressor samples revealed that at a cut-off value ≥5 cells with strong p53 expression (3+ staining) could predict progression to advanced neoplasia with a specificity of 80.2% and a sensitivity of 48.9% (Fig. 5A, B). Moreover, we demonstrate that using a combination of abnormal p53 expression and AGRN BM loss could improve both sensitivity and specificity to identify NDBE patients with an increased risk of developing BE-related neoplasia (Fig. 5C).

Evaluation of AGRN loss among cohort #3

We finally sought to validate our AGRN IHC findings in a multi-institutional cohort #3 of NDBE biopsies from patients who eventually developed neoplasia (Fig. 1). This cohort was composed of 31 patients and the most advanced diagnoses were BE-LGD in 10 (32.3%) patients, BE-HGD in 16 (51.6%) patients and ADCA in 5 (16.1%) patients. The mean AGRN loss in the BM of NDBE glands was 2.24% (mean: 2.24±2.3%, range 0-10% loss). This number for AGRN loss is similar to 2.1±2.8% identified in BE-progressors from cohort

#2 (Supplementary Table S4) with a sensitivity of 67.7% for predicting dysplasia at a cut-off at ≥ 1%.

Discussion

Although endoscopic surveillance and histopathological characterization of BE biopsies remain the standard of care, there are significant challenges to this approach with an unmet need for novel diagnostic and prognostic biomarkers (14,16,39-41) Identifying markers that would help select the small, but clinically important, subset of BE patients who are more likely to progress to cancer and would benefit from intensified endoscopic surveillance is of great interest.

The ECM is a crucial component in tissue organization. All epithelia are underlain by a thin sheet of ECM, called the basal lamina or BM, that provides both mechanical and biochemical support for the epithelial cells, and controls their proliferation, survival and differentiation. Other ECM structures, such as interstitial matrix, play a key role in tissue structure and integrity. The proteins comprising the ECM account for several percent of the mammalian proteome, and several hundred proteins are collectively termed the matrisome (33).

In the current study, our goal was to investigate the expression and distribution of ECM molecules in BE and BE-related neoplasia. Using gene expression analysis, we demonstrated that significant enrichment of ECM matrisome gene sets occurs in human BE and is further enhanced in ADCA compared to normal esophageal controls (Figs. 2, S1-S3, Supplementary Tables S1-3). Although overall ECM levels increase, a more detailed IHC analysis of BM proteins, however, identified an opposite trend and loss of BM AGRN in NDBE biopsies from progressor patients, in BE-related neoplasia (Figs. 3, S4, S5) and in a Levrat model of BE carcinogenesis (Supplementary Fig. S7).

Using IHC, we demonstrated AGRN loss in three distinct BE cohorts to validate its potential utility as a diagnostic (cohort #1) and predictive biomarker (cohorts #2 and 3). BM AGRN loss was significantly higher in BE-HGD and ADCA when compared to NDBE and could distinguish the two with high specificity and sensitivity (Fig. 4). Given the growing interest in identifying reliable biomarkers that can identify BE progression to advanced neoplasia, the potential impact of AGRN is significantly higher in the predictive setting. In a carefully annotated set of NDBE biopsies from progressor and non-progressor patients with long term follow-up (cohort #2) we show that the mean percentage AGRN loss from BM was significantly higher in NDBE samples from progressors compared to non-progressors, and at a cut-off ≥1% could identify patients who progressed to advanced neoplasia with a specificity of 80.2% and sensitivity of 54.8% (Supplementary Fig. S9). This finding was confirmed by evaluating an independent multi-institutional cohort of 31 patients. Collectively, the study highlights the ability of combined IHC assay (p53 and AGRN) targeting disparate proteins to predict future dysplasia in BE.

AGRN is a large multidomain heparan sulfate proteoglycan and is expressed in developing brain and BM of developing organs. Functionally, AGRN was first implicated in the

formation of neuromuscular junctions and in acetylcholine receptor clustering in the central nervous system (42) Although little is known about non-neuronal functions of AGRN, in recent years, a tumor-promoting role has been reported in several cancer types (43-46). Functional studies have shown that AGRN is involved in proliferation, migration and invasion of liver cancer cells by regulating focal adhesion integrity and relaying mechanosensitive signals into cells to regulate YAP activity to promote tumorigenesis (47,48) and to regulate epithelial to mesenchymal transition in pancreatic ductal adenocarcinoma (49). Moreover, Bassat et al. (2017) (50) identified AGRN as an ECM component that promotes cardiomyocyte proliferation and found that AGRN is involved in neonatal heart repair. Clearly AGRN has multiple functional roles in diverse tissues. AGRN has been shown to interact with other ECM proteins, growth factors and cell-surface adhesion receptors, including *NCAM,* integrins, α-dystroglycan and muscle-specific kinase (42).

We recently demonstrated that AGRN is differentially expressed in the muscularis mucosae (MM) underlying sessile serrated lesions (SSL) of the large intestine, providing a sensitive and specific biomarker distinguishing SSL from more common hyperplastic polyps (38). Moreover, in agreement with our current observation on AGRN loss in BE-related neoplasia, we also noted loss of BM-based AGRN reactivity in the dysplastic portion of SSL. To our knowledge, however, the role of AGRN during BE and BE-related neoplasia has not been previously investigated. In this regard, this study demonstrates that AGRN loss from epithelial BM occurs early during the progression from BE to neoplasia and shows its promise as a useful biomarker for diagnosis and prognosis of BE-related neoplasia.

The role of ECM proteins during cancer development is of long-standing and increasing interest, and their aberrant expression during progression of multiple diseases is often associated with poor prognosis (25-27). So far, the ECM has been reported to play a role in several aspects of esophageal carcinogenesis including ECM homeostasis, remodeling and stiffness (51,52). In this context, a diversity of glycoproteins and proteoglycans, such as laminins, fibronectin and tenascin-C as well as matrix metalloproteinases (MMPs) have been reported during BE progression. Dave et al. (2004) (30) used IHC to investigate the distribution of several laminin chains in the BM of the normal upper gastrointestinal tract and Barrett’s metaplasia. Finally, Leppaänen et al. (2017) (28) investigated tenascin-C and fibronectin and showed their upregulation in ADCA when compared to BE and BE-related neoplasia, particularly at the tumor invasive front as compared to the tumor bulk stroma. Although ECM proteins may play an important role during BE carcinogenesis, none of these markers has been used clinically. To date, there have not been any comprehensive and systematic human studies of ECM proteins to evaluate their diagnostic or predictive biomarker utility and their potential to identify those NDBE patients with an increased risk of developing to advanced neoplasia.

Prior efforts at predicting future dysplasia in NDBE samples have focused on DNA content abnormalities, methylation of oncogenes, and inactivation of tumor suppressor genes like p53, although none has been proven efficacious enough to be incorporated into clinical guidelines (53). Among these, p53 IHC appears to be a promising marker for risk stratification in BE (7,20,21,23,24). Nevertheless, its relatively low sensitivity precludes

its use when used in isolation (19) and it has been argued that p53 should constitute one component of a biomarker panel (13). Indeed, recent work has identified two panels of biomarkers in BE to identify future risk of progression and prevalent dysplasia, respectively (54,55), and it has been proposed that the use of three markers in combination, including p53 IHC, can help select patients for prophylactic ablation therapy or intensified endoscopic surveillance (56). Accordingly, we evaluated the utility of AGRN loss in addition to p53 expression and found that assessment of the combination of AGRN loss and abnormal p53 IHC could improve the specificity (86.5%) and sensitivity (58.7%) of identifying NDBE patients with increased risk of developing advanced neoplasia. Further studies are needed to validate our findings in combination with other potential biomarkers to advance their diagnostic and predictive value in order to improve prognosis of future dysplasia for patients with BE.

Study strengths and limits

Our study presents evaluation of a novel biomarker in one of the largest series of BE patients stratified into progressors and non-progressors with long-term follow-up, reviewed by gastrointestinal pathologists with expertise in BE. However, one of the limitations of this study is that most of the patients were part of surveillance programs at tertiary care centers and therefore, the study design likely has an inherent selection bias in terms of evaluating a relatively high-risk population. Having said that, this selection bias at least has the advantage that biopsy sampling was performed using current practice guidelines at advanced endoscopy centers, thus potentially reducing sampling issues related to BE and BE-related neoplasia.

Conclusions

In conclusion, we analyzed the expression patterns of ECM molecules in BE and BE-related neoplasia and show significant enrichment of ECM matrisome gene sets in BE-related dysplasia and ADCA compared to normal esophageal squamous mucosa. Using IHC, we demonstrate that the loss of AGRN in the BM of NDBE glands, either alone, or in combination with p53, can serve as novel biomarkers to identify patients who are at a higher risk of neoplastic progression; albeit with a relatively low sensitivity value of 60%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ADCA	esophageal adenocarcinoma
AGRN	agrin
BE	Barrett’s esophagus
BE-LGD	Barrett’s esophagus with low-grade dysplasia
BE-HGD	Barrett’s esophagus with high-grade dysplasia
BM	basement membrane
COLIV	collagen IV
COLXVIII A1	collagen XVIII, alpha 1
ECM	extracellular matrix
FFPE	formalin-fixed and paraffin-embedded
GSEA	gene set enrichment analysis
HSPG2	heparan sulfate proteoglycan 2 (perlecan)
IHC	immunohistochemistry
NDBE	non-dysplastic Barrett’s esophagus
ROC	Receiver Operating Characteristics

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Translational Relevance

The incidence of Barrett's esophagus (BE)-related adenocarcinoma is increasing, but limitations in current methods lead to failures to identify and triage many patients with high risk disease. There is an unmet need for diagnostic and predictive biomarkers in patients with BE. The role of extracellular matrix (ECM) proteins in progression of BE is largely unknown. In this study, we identified significant enrichment of ECM matrisome gene sets in BE-related neoplasia with selective loss of ECM protein agrin (AGRN). Basement membrane AGRN loss was significantly higher in BE-related high-grade dysplasia (BE-HGD) and esophageal adenocarcinoma (ADCA) when compared to non-dysplastic BE (NDBE) and could distinguish the two with high specificity and sensitivity. Moreover, loss of AGRN in the basement membrane of NDBE glands in combination with an abnormal p53 stain, identified progression to BE-related advanced neoplasia with a specificity and sensitivity of 86.5% and 58.7%.

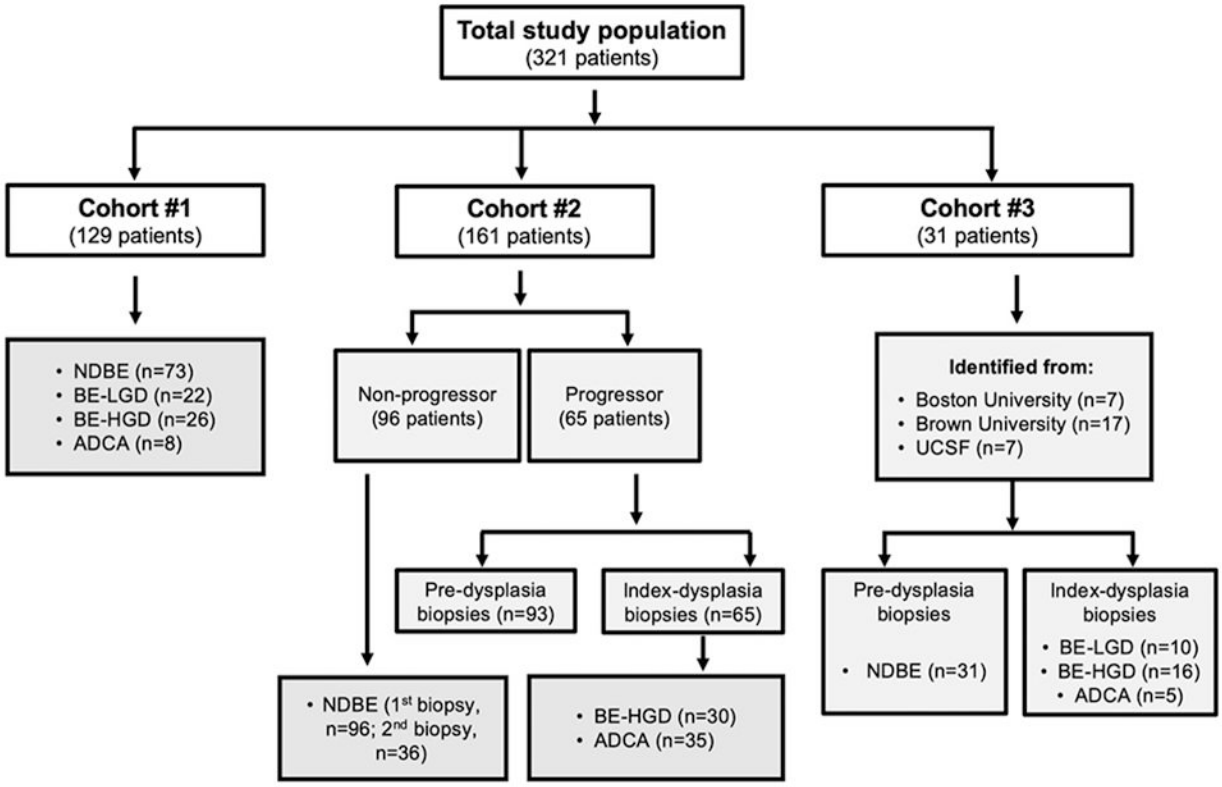


Figure 1. Study summary.

Summary overview of the 3 cohorts and the corresponding individual samples investigated in this study. For details see also the Materials and Methods section. Abbreviations: ADCA, adenocarcinoma; BE, Barrett's esophagus; BE-LGD, Barrett's esophagus with low-grade dysplasia; BE-HGD, Barrett's esophagus with high-grade dysplasia; NDBE, nondysplastic Barrett's esophagus; UC San Francisco

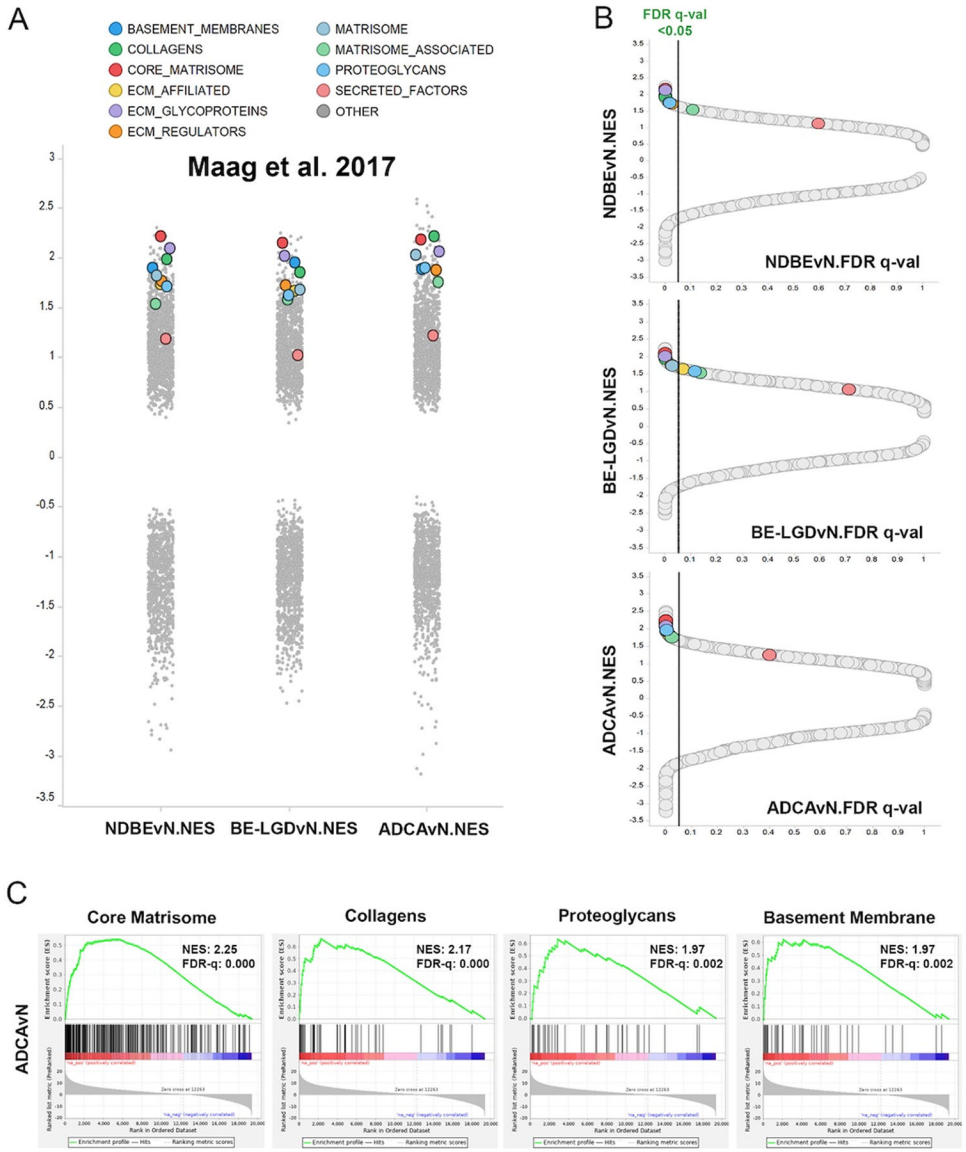


Figure 2. Gene expression analysis of matrisome gene sets during Barrett's esophagus progression (Maag et al. 2017).

Gene expression analysis of matrisome gene sets among many other (non-matrisome) gene sets (msigDB, c2cp; ~2200 sets) during BE progression using RNA-seq data (37) from samples including normal esophagus (N; n=19), NDBE (n=19), BE-LGD (n=8) and ADCA (n=17). Presented are data for the entire matrisome gene set as well as gene sets for individual matrisome subsets: core matrisome genes (including the gene sets: collagens, glycoproteins, proteoglycans and basement membrane) and ECM-associated genes (including the gene sets: ECM regulators, ECM-affiliated and secreted factors). **A)** Strip chart showing normalized enrichment scores (NES) for the comparisons NDBE, BE-LGD and ADCA vs normal esophagus. Matrisome gene sets are indicated as larger and colored points, all other (non-matrisome) gene sets are small grey points. Random jitter has been applied to each strip in order to make overlapping points more visible. **B)** Volcano plots showing each comparison presented in A) separately with NES on the Y axis and False

Discovery Rate (FDR) adjusted q-values on the X axis. The 0.05 FDR q-value significance threshold of 0.05 is indicated by a vertical line. Note the significant enrichment of the entire matrisome gene set (red circles) and of most individual matrisome categories among many gene sets in progressive BE stages, particularly in ADCA compared to normal samples. In contrast, expression of the ECM regulator class (orange) is not markedly increased.
C) Gene set enrichment analysis of core matrisome, collagens, proteoglycans and basement membrane gene sets showing significant enrichment in ADCA relative to normal controls (ADCAvN). For each plot, ADCA is on the left and the normal state is on the right. NES and FDR q-values are indicated in insets for each comparison.

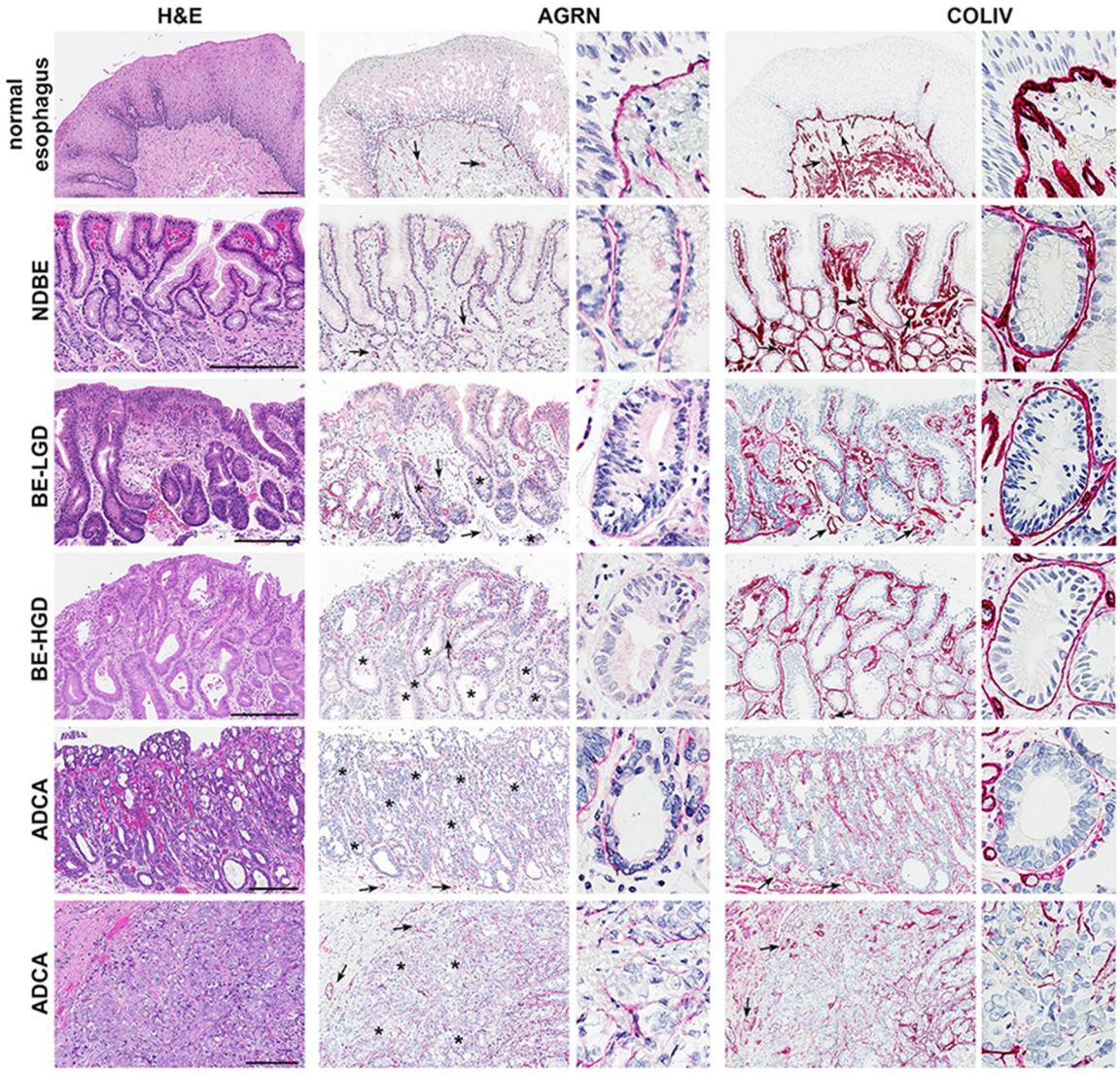
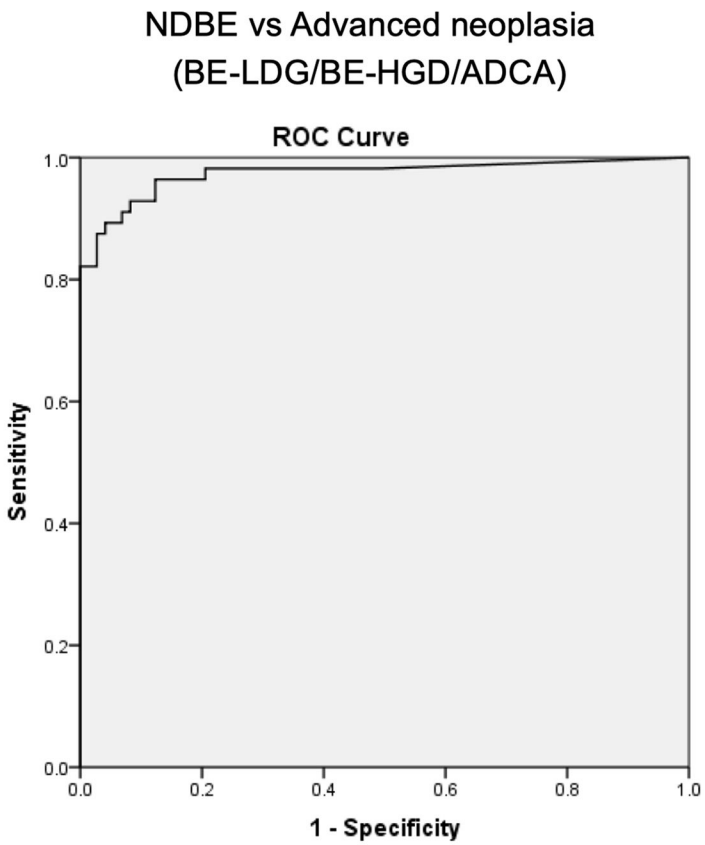


Figure 3. AGRN basement membrane loss during Barrett's neoplastic progression.

Images of representative H&E staining (**left**) as well as IHC for agrin (AGRN, **middle**) and collagen IV (COLIV, **right**) during individual steps of BE-related carcinogenesis. Presented are low magnification and high-magnification images (third and fifth column) of AGRN staining in normal esophagus, NDBE, BE-LGD, BE-HGD and ADCA. Note the positive stain of AGRN and COLIV in the basement membrane (BM) of blood vessels (arrows) at all stages. Note also the presence of AGRN in the BM of normal squamous esophageal epithelium and NDBE glands, and the breaks or loss (*) within the BM of BE-LGD, BE-HGD, and ADCA. In contrast, COLIV is consistently present in the BM of normal esophagus, BE with and without dysplasia and only partially lost in ADCA. Scale bars: 200 μm.

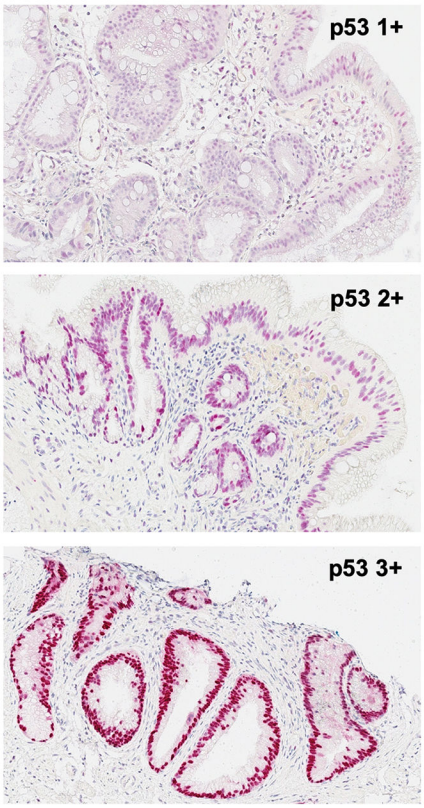


	AUC	Cut-off points	NDBE vs Advanced neoplasia			
			Sensitivity	Specificity	PPV	NPV
AGRN loss (%)	0.951	(≥ 2)	96.4	82.2	80.6	96.8
		(≥ 5)	87.5	95.9	94.2	90.9

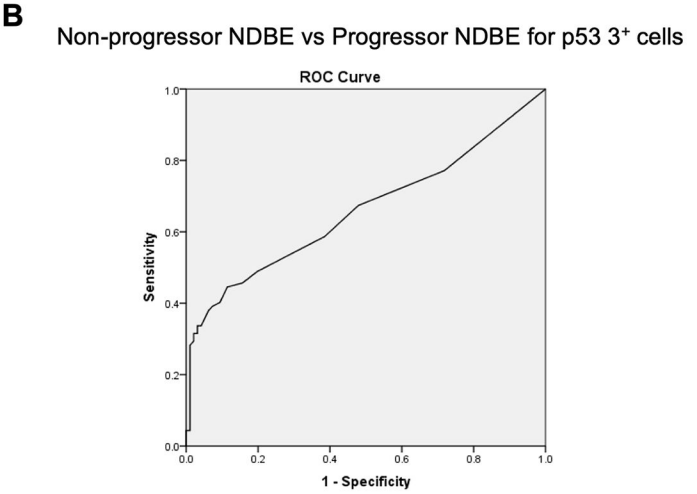
Figure 4. Diagnostic utility of AGRN loss to identify advanced stages of Barrett's esophagus-related neoplasia.

Receiver operating characteristic (ROC) curve analysis of AGRN loss for NDBE compared to all neoplasia (BE-LGD/BE-HGD/ADCA) samples in cohort #1. Shown is the cut-off value ≥2% AGRN BM loss in BE glands as the most appropriate for maximizing both sensitivity (96.4%) and specificity (82.2%). The area under the ROC curve (AUC) was 0.951. Negative predictive values (NPV) and positive predictive values (PPV) are indicated respectively.

A



B



	Cut-off points	Non-progressor NDBE vs Progressor NDBE			
		Sensitivity	Specificity	PPV	NPV
p53 3+	≥ 5 cells	48.9	80.2	70.3	62.1
	≥ 10 cells	38	93.8	85.4	61.2

C

p53 3+ & AGRN loss combined	P53 ≥ 10 cells AGRN ≥ 2% loss	58.7	86.5	80.6	68.6
	P53 ≥ 5 cells AGRN ≥ 1% loss	72.8	64.6	66.3	71.3

Figure 5. Utility of p53/AGRN combination to identify the progression to advanced Barrett's esophagus-related neoplasia.

A) Images of representative p53 IHC staining in BE biopsies indicating the increased nuclear staining of p53 [1+ (weak), 2+ (medium) and 3+ (strong) p53 positive cells, see Materials and Methods for details].

B) ROC curve analysis of p53 IHC for NDBE progressor samples compared to NDBE non-progressor samples in cohort #2. Shown is the cut-off value ≥5 strong p53 (3+) positive cells as the most appropriate for maximizing both sensitivity (48.9%) and specificity (80.2%).

C) Presented are also the cut-off points and values for p53 and AGRN combined analysis. Note the additional improvement of sensitivity and specificity using p53 and AGRN in combination.

Negative and positive predictive value (NPV and PPV) are indicated respectively.

Table 1.

Clinicopathologic features and AGRN loss in samples of the primary cohort #1.

Presented are the clinicopathological features for patients (n=129) and the percentage basement membrane AGRN loss and analysis for the initial cohort #1 representing the following pathologic diagnoses: NDBE (n=73), BE-LGD (n=22), BE-HGD (n=26), and ADCA (n=8). Also presented are the mean ± SD and range of percentage basement membrane AGRN loss in glands of NDBE samples compared to neoplasia (BE-LGD/BE-HGD/ADCA) biopsies. Categorical parameters were analyzed using Chi squared test or Fisher's Exact test and continuous parameters (variables) were analyzed using Mann-Whitney U test. For comparisons, Student's two-tailed t test was used. SD, standard deviation.

Clinical parameters (Number of patients n=129)	NDBE (n=73)	BE-LGD (n=22)	BE-HGD (n=26)	ADCA (n=8)	P value
Gender					
Male	53 (72.6%)	17 (77.3%)	23 (88.5%)	6 (75%)	0.430
Female	20 (27.4%)	5 (22.7%)	3 (11.5%)	2 (25%)	
Mean age in years ± SD, Median	64.7±9.3, 66	69.5±9.9, 68.5	62.1±10.5 62.5	61.3±13.7, 60	0.781
Length of BE (n=99)					
Short	37 (61.7%)	3 (20%)	5 (27.8%)	3 (50%)	0.005
Long	23 (38.3%)	12 (80%)	13 (72.2%)	3 (50%)	
AGRN loss (%)					
Mean ± SD, Range	0.9±1.5, 0-5.8	19.1±14.5, 3.3-45.8	16.1±14.5, 0-66.6	45.8± 26.5, 14.1-78.4	<0.001

Table 2.

Comparison of clinicopathological features of non-progressor (N=96) and progressor (N=65) patients in cohort #2.

Presented are the clinicopathological features for patients (n=161) in the non-progressor (n=96, patients, n=96 1st biopsies) and progressor groups (n=65 patients, n=93 biopsies) in cohort #2 based on the initial diagnosis. In the non-progressor group, the mean duration between the index Barrett's biopsy and the most recent biopsy was 10.7 years. In contrast, within the progressor group, the mean duration between the non-dysplastic biopsy and the outcome neoplastic biopsy was 4.5 years. Categorical parameters were analyzed using Chi squared test or Fisher's Exact test and continuous parameters (variables) were analyzed using Mann-Whitney U test. BMI, body-mass index.

Clinicopathological parameters (patients n=161)	BE non-progressors (n=96 patients)	BE progressors (n=65 patients)	P value
Gender			
Male	62 (64.6%)	56 (86.2%)	0.002
Female	34 (35.4%)	9 (13.8%)	
Mean age in years at endoscopic and histologic diagnosis of BE ± SD, Range	56.5±10.6, 33-80	63.4±12.2, 29.5-87.1	<0.001
BMI (n=127), Mean ± SD, Range	28.8±5.5, 19.6-49.6	28.9±6.3, 17-48.2	0.922
History of smoking (n=126)			
Smokers	46 (63.9%)	38 (70.4%)	0.445
Non-smokers	26 (36.1%)	16 (29.6%)	
History of alcohol intake (n=121)			
Yes	37 (52.1%)	23 (46%)	0.508
No	34 (47.9%)	27 (54%)	
Mean length of Barrett's epithelium in cm (n=152) ± SD, Range	2.5±2.8, 0.5-15.0	5.2±3.7, 0.5-15	<0.001
Endoscopic type of BE			
Long BE (≥3cm)	31 (34.1%)	46 (75.4%)	<0.001
Short BE (<3cm)	60 (65.9%)	15 (24.6%)	
Highest grade of neoplasia	BE	HGD, n=30 (46.2%) ADCA, n=35 (53.8%)	-
NDBE follow-up from BE till progression to HGD/ADCA * or NDBE ** ± SD, Range	10.7±4.4, 2-20	4.5±4.5, 1-19	-

* for progressors

** for non-progressors

Table 3.
Comparison of initial vs consensus diagnosis and AGRN loss in cohort #2.

Presented are the comparisons of the initial diagnosis (A, n=272) and the consensus/majority (2 of 3) diagnosis based on blinded validation for AGRN loss by 3 gastrointestinal pathologists (B, n=242) for cohort #2 as well as the mean ± SD and range of percentages of basement membrane AGRN loss for the individual samples. Note that the AGRN loss is predominantly seen in advanced neoplasia biopsies in the progressor group compared to the non-progressor group.

A

Initial diagnosis (n=272)	AGRN loss (%)	
	Mean±SD	Range
NDBE non-progressor 1st (n=96)	0.5±1.2	0-8.3
NDBE non-progressor 2nd (n=36)	0.5±0.8	0-3.2
NDBE progressor (93)	2.1±2.8	0-15.2
BE-HGD progressor (18)	29.5±27.1	1.1-86.7
ADCA progressor (29)	41.6±25.4	1.3-97.9

B

Consensus diagnosis (n=242)	AGRN loss (%)	
	Mean±SD	Range
NDBE non-progressor 1st (n=95)	0.6±1.2	0–8.3
NDBE non-progressor 2nd (n=34)	0.5±0.8	0–3.2
NDBE progressor (n=68)	1.6±2.7	0–15.2
BE-LGD progressor (n=2) = initial HGD	62.2±34.6	37.7–86.7
BE-HGD progressor (n=26)	26.8±23.8	1.1–86.2
ADCA progressor (n=17)	49.7±24.9	12.6–97.9