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Research Article

Cancer Prevention Research

Goblet Cell Ratio in Combination with Differentiation and Stem Cell Markers in Barrett Esophagus Allow Distinction of Patients with and without Esophageal Adenocarcinoma

Raphael Schellnegger[1], Anne Quante[2,3], Susanne Rospleszcz[4], Martina Schernhammer[1], Bettina Höhl[1], Moritz Tobiasch[1], Agnieszka Pastula[1], Anna Brandtner[1], Julian A. Abrams[5], Konstantin Strauch[2,3], Roland M. Schmid[1], Michael Vieth[6], Timothy C. Wang[5], and Michael Quante[1]

Abstract

The increasing incidence of esophageal adenocarcinoma (EAC) is mirrored by the increasing prevalence of Barrett esophagus, a precursor lesion resulting in a large number of individuals "at risk" for this lethal malignancy. Among patients with Barrett esophagus, only about 0.3% annually will develop EAC. Because large numbers of patients are followed in endoscopic surveillance, there is a need for risk prediction among a growing population of patients with Barrett esophagus. We identified four potential biomarkers from an inflammation (IL1β)-dependent mouse model of Barrett esophagus and tested them in 189 patients with Barrett esophagus with and without high-grade dysplasia (HGD)/early cancer (T1). The primary goal was to distinguish patients with Barrett esophagus with no evidence of dysplasia from those with dysplasia. Increasing stem cell marker LGR5 and niche cell marker DCLK1 and decreasing differentiation marker (secretory mucus cells, TFF2$^+$ cells) correlated with elevated tumor score in the mouse. Having outlined the origin of those markers in the Barrett esophagus mouse model, we showed the applicability for human Barrett esophagus. We compared 94 patients with nondysplastic Barrett esophagus tissue with 95 patients with Barrett esophagus and HGD or early cancer. Low levels of TFF2 (AUC 87.2%) provided the best discrimination between nondysplastic Barrett esophagus and Barrett esophagus with cancer, followed by high levels of DCLK1 (AUC 83.4%), low goblet cell ratio (AUC 79.4%), and high LGR5 (AUC 71.4%). The goblet cell ratio, rather than the presence of goblet cells per se, was found to be an important discriminator. These findings may be useful in developing future risk prediction models for patients with Barrett esophagus and ultimately to improve EAC surveillance. *Cancer Prev Res; 10(1); 55–66. ©2016 AACR.*

Introduction

Barrett esophagus is a premalignant condition defined by replacement of the squamous epithelium in the distal esophagus by specialized intestinal metaplasia (1). The development of Barrett esophagus is thought to represent the initial step in the histopathologic progression to low-grade as well as high-grade dysplasia (HGD) and esophageal adenocarcinoma (EAC; ref. 2). The incidence of EAC has increased at a relative rate of 4% to 10% annually and about 460% in 30 years in regions of the Western world (3) and the cancer has still a very poor prognosis with a mean 5-year survival rate of less than 20%. The prevalence of the precursor lesion, Barrett esophagus, has also concordantly increased greatly over the last decades, resulting in a large number of individuals "at risk" for this very lethal malignancy. Nevertheless, accurate historical assessments of Barrett esophagus rates are difficult to estimate and therefore one might speculate that factors other than the presence of intestinal metaplasia are correlating with risk assessment and increased EAC rates. While close monitoring of patients with Barrett esophagus would in theory reduce EAC mortality, only about 0.2% to 0.3% per year will eventually develop EAC (4). A large number of patients are therefore kept under endoscopic surveillance for the detection of a relatively small number of cancers. It seems clear that there is a critical need to develop better preventive strategies, possibly by risk stratification and identification of high-risk Barrett esophagus subsets that would benefit from targeted intervention.

Regular endoscopic surveillance of patients with Barrett esophagus does offer the opportunity for early intervention, as the neoplasm in theory can be detected and resected at a potentially curable stage. However, the development of an optimal surveillance strategy for patients with Barrett esophagus has been

[1]II. Medizinische Klinik, Klinilkum rechts der Isar, Technische Universitaet München, Munich, Germany. [2]Institute of Medical Informatics, Biometry and Epidemiology, Chair of Genetic Epidemiology, Ludwig-Maximilians-Universitaet, Munich, Germany. [3]Institute of Genetic Epidemiology, Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany. [4]Institute of Epidemiology II, Helmholtz Zentrum München - German Research Center for Environmental Health, Neuherberg, Germany. [5]Department of Medicine and Irving Cancer Research Center, Columbia University Medical Center, New York, New York. [6]Klinikum Bayreuth, Institut für Pathologie, Bayreuth, Germany.

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Corresponding Author: Michael Quante, II. Medizinische Klinik, Klinikum rechts der Isar, Ismaninger Str. 22, Room 502.3.08, München 81675, Germany. Phone: 49-89-41407870; Fax: 49-89-41406796; E-mail: Michael.Quante@tum.de

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restricted by the lack of a tractable preclinical model of Barrett esophagus and EAC.

We previously established a transgenic mouse model in which IL1β expression was targeted to the esophageal and squamous forestomach mucosa. These mice exhibit spontaneous esophagitis, followed by progression to Barrett esophagus and EAC (5). In contrast to most human Barrett esophagus disease, the Barrett esophagus mouse model has a lower incidence of classic intestinal metaplasia but on the other hand a high rate of progression to EAC. In addition, consistent with the first description of this disease by Barrett and colleagues (6–8), careful analysis of the Barrett esophagus mouse model revealed that the metaplastic lesions originate from the gastric cardia, particularly from $LGR5^+$ progenitor cells (5, 9–11). In response to esophageal inflammation, the $LGR5^+$ epithelial progenitors appear to migrate from the gastric cardia into the squamous esophagus, and in both human and murine Barrett esophagus, $LGR5^+$ progenitors were strongly associated with the development of dysplasia (12, 13). Furthermore, we could demonstrate that prior to the development of intestinal metaplasia, a gut-like columnar-lined epithelium (CLE) from a regenerative cell lineage expressing TFF2 and CDX2 appears in the esophagus (14–18).

In this translational study, we apply findings from the Barrett esophagus mouse model to human tissue and evaluate 4 specific biomarkers (goblet cell ratio, TFF2, LGR5, DCLK1) to distinguish the characteristics of metaplasia that is associated with cancer. We suggest that this novel risk prediction model shows great promise and might contribute to individual-based risk stratification in Barrett esophagus surveillance programs.

Materials and Methods

Animal studies

Mice were allowed a standard chow diet from birth until weaning, and water *ad libitum* in groups of less than 5 animals. Once per week, the animals are transferred to a fresh cage under a transfer station. Water bottles are changed weekly, all animal experiments were approved by the District Government of Upper Bavaria and performed in accordance with the German Animal Welfare and Ethical Guidelines of the Klinikum rechts der Isar, TUM, Munich, Germany. The Dclk1-CreERT2 transgenic mice were crossed to Rosa26R-Tomato/GFP reporter strains as previously described (19). Human IL1β transgenic mice (Barrett esophagus mouse model) generated in our laboratory by targeting expression of IL1β to the esophagus using the Epstein-Barr virus L2 promoter have been previously described. The mice show chronic esophagitis and progress over time (∼12 months) to metaplasia and dysplasia (5). All transgenic mice were on a pure C57/B6 background after 6 backcrosses. C57/B6, LgR5-CreTM-IRES-GFP, Rosa26R-LacZ mice were purchased from Jackson Laboratories Inc.

Paraffin sections fixed in 10% formalin were incubated with primary antibodies: DCLK1 (Abcam 1:200), TFF2 as previously described (20), and control IgG2a. Biotinylated secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) and ABC avidin-biotin-DAB detection kit (Vector Labs) were used for detection and visualization according to the manufacturer's protocol.

The stomach and esophagus from transgenic and control mice were fixed in 10% formalin, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin (H&E), Periodic acid Schiff reaction (PAS), as well as Alcian blue. The area of mucus-producing cells (i.e., goblet-like cells) versus the non–mucus-producing columnar cells was evaluated in the overall metaplastic region of the Barrett metaplasia at the squamocolumnar junction (SCJ) semiquantitatively by adapting a previously established scoring system (5). The percentage of mucus-producing cells within the whole epithelium was calculated.

Human study population

To analyze the 4 biomarkers in human Barrett esophagus, we collected a total of 189 specimens from a tertiary community hospital pathology department in Germany (Klinikum Bayreuth, Institut für Pathologie, Bayreuth, Germany). All samples were identified by a pathology database search that included patients from January 2008 to May 2013. The Ethics Committee of TU-Munich approved the study protocol. Inclusion criterion was diagnosed Barrett esophagus with intestinal metaplasia including goblet cells at any site on either biopsies or endoscopic mucosal resection (EMR) and an EAC UICC stage < pT2; there was no limitation on age. Subjects with a history of additional malignancy or EAC treatment other than EMR were excluded, there was no limitation on age. Including only EMR specimens was done to reduce a tumor-specific field effect on the remaining tissue, which we assumed would be minimized by choosing T1 EMR specimens only.

The study used a case–control design with 2 groups of approximately equal numbers of patients. The one (nondysplastic Barrett esophagus) group included endoscopic biopsies from 94 patients who according to records never showed no signs of dysplasia/EAC at any time point. The other (Barrett esophagus associated with EAC) group consisted of 95 primarily EMR samples with Barrett esophagus and HGD or early cancer simultaneously. Patients with only low-grade dysplasia were excluded in advance to avoid issues of diagnostic accuracy (21). We were considering all available esophageal material for a patient available for analysis. In cases where only endoscopic biopsy material was used, all available patient material (at least 3 different biopsy sites) were taken into consideration to get comparable results with EMR specimens.

Evidence of reflux in adjacent squamous cell epithelium was evaluated by scoring 0 to 3 according to no-, mild-, moderate-, and severe- hyperregenerating esophagopathia. As not all of the specimens obtained were in perfect condition, we only included those with sufficient nonlacerated tissue available. The numbers of analyzed samples were detailed in every figure.

H&E/Immunohistochemistry

All staining was performed on 5-μm paraffin sections. Standard H&E and Alcian Blue protocols, as well as (PAS) reaction were used for evaluation of goblet cell density. For immunohistochemical (IHC) staining, incubation was performed using primary antibodies: DCLK1 (Abgent Inc., 1:200), TFF2 (Proteintech Group, 1:1,000), and LGR5 (Abcam, 1:100). Biotinylated secondary antibodies (Jackson ImmunoResearch) were diluted (1:200) in 2% BSA/PBS and incubated for 1 hour each at room temperature.

The mounted slides were deparaffinized in xylene and rehydrated through descending concentrations of ethanol. Antigen retrieval was performed using a citrate buffer heated in a pressure cooker for 5 minutes and then cooled to room temperature. Blocking of endogenous peroxidases was accomplished by incubating sections in 3% hydrogen peroxide for 10 minutes and in

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5% goat serum for 30 minutes. The prepared primary antibodies were incubated with sections overnight at 4°C. Avidin/Biotin detection kit (Vector Labs) was used according to the manufacturer's protocol. Sections were counterstained with hematoxylin, dehydrated in ascending concentrations of ethanol followed by clearance with xylene, and coverslipped permanently for light microscopy. Negative controls were obtained by excluding the primary antibody.

Histopathologic analysis and IHC scoring

All human specimens were examined by R. Schellnegger and M. Vieth (specialist in gastrointestinal pathology) without knowledge of the clinical report. Scoring of IHC was based on consensus opinion. Goblet cell density was calculated separately by the 2 independent investigators and mean values were used to calculate the final goblet cell ratio. Bright field microscopy was done on a Zeiss Axio microscope with low-power view; images were captured on an Axio-Cam HRc and analyzed with AxioVision Software for Windows (Carl Zeiss AG).

As we did not intend to compare intestinal metaplasia with EAC, we were selecting all areas of nondysplastic intestinal metaplasia for both groups. The transition zone from intestinal metaplasia to dysplasia/EAC was marked and dysplastic regions not taken into account. In addition, in 10 EAC (T1) containing EMR specimens, we performed a separate evaluation of histopathologic marker and goblet cell ratio in a very close area to dysplasia at a distance <3 mm and in a more distant area (>3 mm) to evaluate tumor-specific effects in the close neighboring tissue. The goblet cell density was calculated by the following method: every lumen surrounded by columnar-lined cells was assumed to represent one Barrett crypt. Each crypt was scored as (+) or (−) for goblet cells. The goblet cell ratio was then defined as the number of (+) crypts divided by the total number of crypts. Every investigator calculated mean data of 3 random low-power fields. To address the issue with pseudo–goblet cells (22), different staining was compared and submucosal Brunner glands, as well as lumen open to the surface, were not taken into account.

For scoring of IHC, a semiquantitative approach [i.e., modified immunoreactive score (IRS) described by Remmele and colleagues (ref. 23)] was used. Again, only areas of nonmalignant Barrett esophagus were evaluated; the highest scoring per patient specimen (i.e., whole EMR or all available patients' biopsies) was used to define the final score. Staining in the submucosal glands and the epithelial surface was again ignored. Whenever a single Barrett crypt showed cells with positive staining, it was considered as being positive (+). The percentage of (+) crypts was used to generate the final score. For the stem cell marker LGR5 and the niche cell marker DCLK1, staining scores were adapted from prior Barrett esophagus analyses (24). The scale ranged from 0 to 3, where 0 represented <10%, 1 represented 10% to 30%, 2 represented 30% to 60%, and 3 represented > 60% of positive crypts within the Barrett esophagus area. The scale used for TFF2 scoring was based on a modified Allred score previously used in Barrett esophagus (25), with a scale from 0 to 4, where 0 meant <1%, 1 meant <25%, 2 meant <50%, 3 meant <75%, and 4 meant >75% of positive crypts within the Barrett esophagus area.

Statistical analysis

In the mouse model, we used ANOVA to score the significance of mucus cell ratio, TFF2, DCLK1, and LGR5 in correlation with the tumor score (range, 1–4). In human tissue, we used the *t* test to formally evaluate whether the mean of the goblet cell ratio from the nondysplastic Barrett esophagus group differed significantly from the Barrett esophagus with EAC cohort. To test whether the distribution of TFF2, LGR5, or DCLK1 scores in humans differed significantly between both groups, we used the χ^2 test. $P < 0.05$ was considered statistically significant. A logistic regression model was set up separately for each biomarker (goblet cell ratio, TFF2, LGR5, and DCLK1) to determine the effect of each marker on the outcome of a subject (nondysplastic Barrett esophagus vs. Barrett esophagus with EAC). Additional models that included combinations of the biomarkers were generated. For each model, we assessed its ability to discriminate between nondysplastic Barrett esophagus and Barrett esophagus with EAC by estimating each model's area under the receiver operator characteristic (ROC) curve (AUC). This measure ranges from 0.5 (no discriminative ability) to 1 (perfect discrimination). Furthermore, we determined the true-positive rates (sensitivity) and true-negative rates (specificity) by calculating a ROC curve. The optimum cutoff value was determined by exploring the point of the ROC curve for which Youden index reaches its maximum. We used R v3.2.1 with package "pROC" for these calculations (26, 27).

Results

A high goblet cell ratio correlates with Barrett metaplasia but not adenocarcinoma

While goblet cells have primarily been used as a marker of intestinal metaplasia, we and others have proposed that goblet cells are terminally differentiated cells with reduced ability to transform into malignant cells (5, 12, 28, 29). Here, we analyzed the abundance of mucus-producing (i.e., goblet-like) cells in pL2-IL1β mice with or without tumors as a surrogate for goblet cell–like differentiation. True goblet cell differentiation is rarely seen in mouse models of upper gastrointestinal metaplasia/dysplasia, therefore we propose that extensive mucus differentiation could be assumed to represent the murine equivalent of intestinal metaplasia seen in human Barrett esophagus. PAS and Alcian blue–positive mucus-producing cells were counted in regions of Barrett esophagus metaplasia at the SCJ in 9- and 12-month-old pL2-IL1β mice. Tumors were scored as described previously (5) and correlated with the amount (%) of mucus-producing cells in the total Barrett esophagus region in the mouse model. Indeed, we could demonstrate that a decreased ratio of goblet-like cells within the area of Barrett esophagus tissue at the SCJ significantly (ANOVA: $P < 0.0001$) correlated with an increased tumor score (from 1 to 4, Fig. 1A and B) at 2 different stages in the pL2-IL1β mouse model. On the basis of this data from the Barrett esophagus mouse model, we hypothesized that an increased ratio of goblet cells within the intestinal metaplasia of human Barrett esophagus tissue might also correlate with a reduced cancer risk. Because dysplasia is associated with a loss of goblet cells, it was of interest to correlate the goblet cell ratio with the differentiation status of the tissue. Using a case–control study design, we compared the goblet cell ratio in patients with nondysplastic Barrett esophagus with the goblet cell ratio in patients with Barrett esophagus with T1 noninvasive cancer in EMR material, focusing in the latter on nondysplastic Barrett esophagus tissue adjacent to the cancer site. Analysis of the cohorts' characteristics revealed that both groups were predominantly male, with 69% and 84% men in the Barrett esophagus and EAC groups, respectively. There was no significant difference in age between the 2 groups (Table 1). No correlation of

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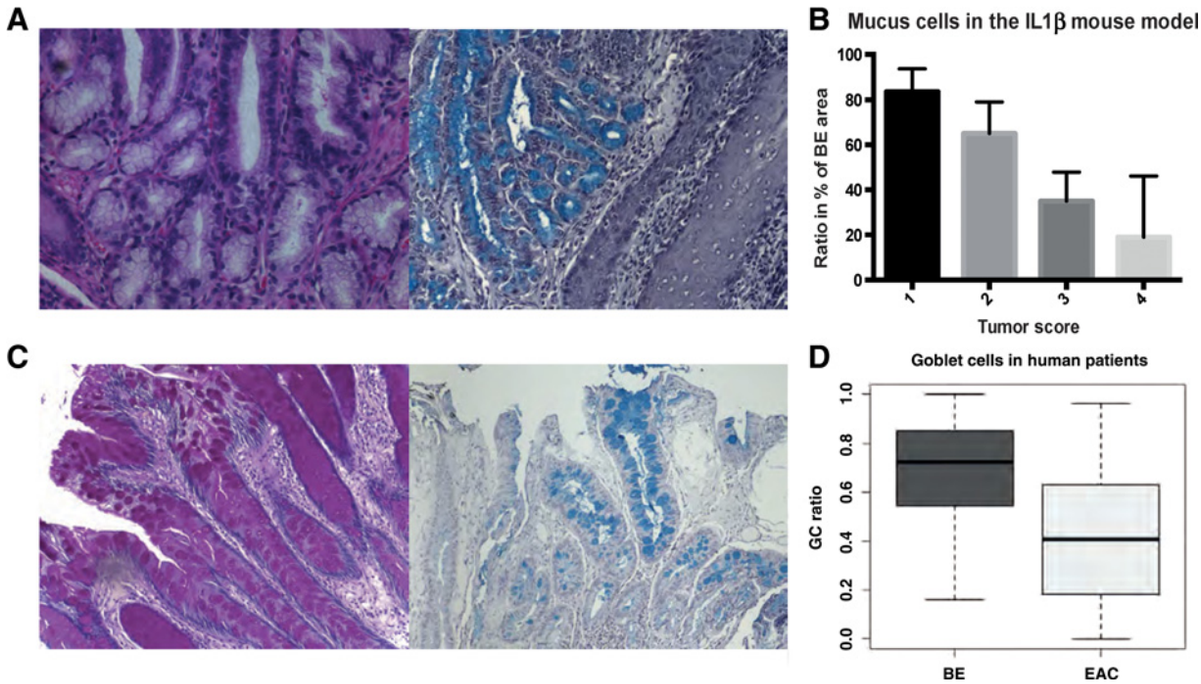

Figure 1.

A, PAS and Alcian blue staining of murine Barrett esophagus metaplasia in the cardia showing mucus-producing cells. **B,** Correlation of mucus cell ratio (%) with the tumor score; (%) of area containing mucus-producing cells was counted in the metaplastic and dysplastic area of the mouse forestomach (ANOVA: $P < 0.0001$, R^2: 0.6951, F: 39.52). **C,** PAS and Alcian blue staining of human Barrett esophagus metaplasia showing specialized intestinal metaplasia with goblet cells. **D,** Boxplot of the goblet cell ratio in human biopsies from Barrett esophagus or adjacent to EAC tissue with bars marking mean values (0.69 vs. 0.41 in Barrett esophagus and EAC respectively, $P < 0.0001$) and whiskers defining range from Min to Max.

goblet cell ratio with age or gender was found (data not shown). Notably, the goblet cell ratio differed significantly ($P < 0.0001$), with a mean goblet cell ratio of 0.69 in the 94 patients with nondysplastic Barrett esophagus and 0.41 in the 95 patients with Barrett esophagus and cancer group (Fig. 1C and D). To exclude tumor-specific effects in the tumor containing EMR specimens, a second evaluation in a more closely adjacent or more distant part of the EMR specimen was performed and did not show a difference in goblet cell ratio (and all other markers as described below) between close (<3 mm) and distant (>3 mm) tissue locations (Supplementary Fig. S1), indicating that the observed analysis is indeed reflecting a field- and patient-specific effect with the Barrett esophagus tissue rather than a localized phenomenon. Thus, in this human cohort, an elevated goblet cell ratio could discriminate between Barrett esophagus with and without adjacent dysplasia.

TFF2 expression correlates with decreased risk of dysplasia

TFF2, previously known as spasmolytic polypeptide, is a small peptide normally expressed in the stomach and duodenum but upregulated throughout the gastrointestinal tract in the setting of injury and inflammation (18). While TFF2 is not expressed in the normal esophageal squamous epithelium, the presence in TFF2-expressing cells at the base of Barrett glands has been reported by several groups (12, 13). In the Barrett esophagus mouse model, TFF2 is concordantly absent in esophageal squamous epithelium but abundant in the gastric cardia of IL1β mice (Fig. 2A) and wild-type (WT) mice (data not shown). Interestingly, TFF2 was found to be highly upregulated (ANOVA: $P < 0.0001$) in the lower esophagus of L2-IL1β mice with Barrett-like metaplasia but was downregulated (ANOVA: $P < 0.0001$) in older (9- and 12-month-old) pL2-IL1β mice with dysplasia and tumors (Fig. 2A and B). Therefore, we hypothesized that TFF2 functions as a marker of nondysplastic Barrett metaplasia, with decreased TFF2 expression representing an increased risk of malignant progression.

Indeed, in our human cohort, a significant upregulation of TFF2 was present in the nonmalignant Barrett esophagus compared with the EAC group ($P < 0.0001$). In patients with nondysplastic Barrett esophagus, 87% had a score of 2 or more, whereas in patients with Barrett esophagus with cancer, 72% had values of 0 or 1 (Fig. 2C). IHC showed that TFF2 was specifically expressed in epithelial cells mainly at the base of the Barrett glands; the surface layer or upper part of the glands did not show any TFF2 positivity (Fig. 2D). Among nondysplastic Barrett glands, few showed a complete absence of TFF2, indicating the nearly ubiquitous presence of TFF2 in nondysplastic Barrett esophagus glands (18). TFF2 staining could also be found in adjacent gastric cardia or corpus tissue but was not detected in esophageal squamous

Table 1. Age distribution did not vary and was conform with literature; both groups were associated with male gender (OR, 0.318; $P = 0.0013$)

	Number of patients	Mean age (±SD), y	Male sex (%)
Barrett esophagus	94	62 (±12)	69%
EAC	95	61 (±12)	84%

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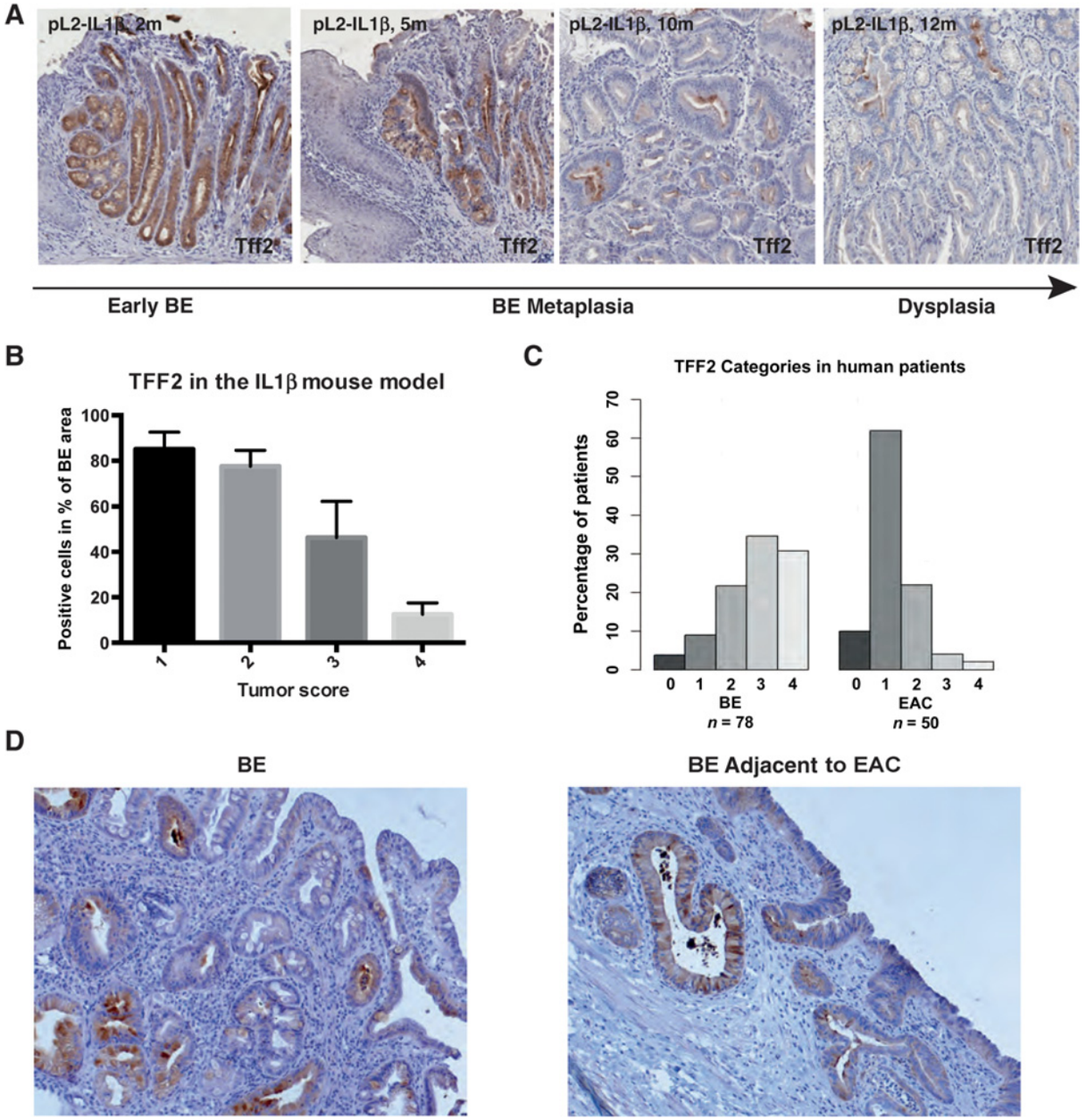

Figure 2.

A, TFF2 IHC of Barrett esophagus metaplasia in the pL2-IL1β mouse model with increasing status of early (2 months) to metaplastic (5 and 10 months) Barrett esophagus and dysplasia (12 months). **B,** Histopathologic score in correlation with TFF2 staining in the pL2-IL1β mouse model (ANOVA: $P < 0.0001$, R^2: 0.8728, F: 54.91). **C,** TFF2 staining in human biopsies from Barrett esophagus or adjacent to EAC tissue was scored from 0 to 4, percentage of each score in both groups is shown (4%/9%/22%/35%/31% in Barrett esophagus as well as 10%/62%/22%/4%/2% in EAC). **D,** Representative TFF2 staining of human biopsies from Barrett esophagus or adjacent to EAC tissue.

epithelium and there was no difference between close (<3 mm) and distant (>3 mm) tissue locations (Supplementary Fig. S1).

The stem cell marker LGR5 can discriminate between Barrett esophagus and EAC-associated Barrett esophagus

LGR5/GPR49, a leucine-rich orphan G-protein–coupled receptor, was shown to specifically label stem cells in the intestine, the so-called crypt-based columnar (CBC) cells (30, 31). In the L2-IL1β mouse model, we previously demonstrated that LGR5-expressing cells also function as stem cells in the gastric cardia and can serve as potential cells of origin for Barrett esophagus and dysplasia (5). Here, we show through lineage tracing that LGR5$^+$ cells can give rise to polyclonal metaplastic and dysplastic Barrett esophagus crypts at the SCJ in IL1β mice crossed to Lgr5-CreERT2

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mice and Confetti reporter mice (Fig. 3A). L2-IL1β;Lgr5-CreERT2; Confetti mice were induced with tamoxifen at 3 months of age, a time point where Barrett esophagus metaplasia containing increased LGR5 cells was present. Analysis of lineage tracing at 12 months of age, 9 months after Cre induction, confirmed the strong lineage relationship between LGR5$^+$ cells in the cardia and the development of metaplastic and dysplastic tissue at the SJC (Fig. 3A). In particular, the use of Confetti mice allowed us to demonstrate that multiple different LGR5$^+$ cells within the Barrett esophagus tissue contribute to Barrett esophagus and EAC, as we observed at 9 months postinduction, the presence of crypts with at least 2 different colors (Fig. 3A). While we were not able to analyze a detailed time course of the development of Barrett esophagus clonal heterogeneity, the data were clear that multiple LGR5 stem cells present early on sustain Barrett esophagus crypts over time. In addition, we were able to demonstrate significant correlation (ANOVA: $P < 0.0001$) between the number of LGR5$^+$ cells and the tumor score in the Barrett esophagus mice, suggesting a possible direct contribution of LGR5$^+$ cells to accelerated dysplasia (Fig. 3B).

In our human patient cohort, IHC demonstrated LGR5 expression near the base of the glands (Fig. 3D). Almost no staining was visible at the surface. The LGR5$^+$ cells were clustered and not spread singularly over the whole gland, concordant with the mouse data, suggesting the presence of a stem cell niche present in the lower third of the glands (12, 13). LGR5$^+$ cells were found in both nondysplastic Barrett esophagus and in Barrett esophagus tissue adjacent to EAC. However, the distribution of LGR5 staining categories in nondysplastic Barrett esophagus samples was significantly lower than in those of the Barrett esophagus with EAC group ($P = 0.02$). The LGR5 score showed a significant increase in Barrett esophagus tissue adjacent to EAC, that is, 41% of the 44 EAC specimens had a LGR5 score of 3, compared with only 13% from the 40 Barrett esophagus specimens (Fig. 3C). LGR5 was not expressed in any esophageal tissues other than columnar–epithelial cells and in particular was absent in squamous epithelial cells (Fig. 3B), and there was no difference between close (<3 mm) and distant (>3 mm) tissue locations (Supplementary Fig. S1).

DCLK1 identifies predysplastic tissue

DCLK1 is a microtubule-associated kinase expressed in isolated cells in the stomach, intestine, and colon (19). Using Dclk1-CreER^{T2} transgenic mice, we recently demonstrated that a subpopulation of intestinal DCLK1$^+$ tuft cells is exceptionally long-lived and appeared to regulate intestinal stem cell function. These long-lived DCLK1$^+$ cells are expanded in chronic inflammation and in the setting of preneoplasia and can also function as cancer-initiating cells in colon cancer (19). Similar to observations in the inflamed colon, DCLK1$^+$ cells were significantly (ANOVA: $P < 0.0001$) amplified in regions of metaplasia in our mouse model of Barrett esophagus and EAC (Fig. 4A). We observed a further increase in more advanced Barrett esophagus with low-grade dysplasia (Fig. 4A and D). However, as seen in other cancers, DCLK1$^+$ cells disappeared with the development of severe dysplasia or a tumor score of 4 (Fig. 4D) and therefore seem to serve as a marker for preneoplasia. In particular, we confirmed that long-lived DCLK1$^+$ stem cells accumulate in the inflamed gastric cardia of the mouse (Fig. 4B) and expand throughout the Barrett esophagus glands, as shown using L2-IL1β;Dclk1-CreER^{T2}; Rosa26r-LacZ mice induced at 3 months and examined at 9 months (Fig. 4B). Of note, in this setting, no lineage tracing of the crypts from Dclk1$^+$ cells was detected under these conditions.

In the human cohort, DCLK1$^+$ cells were located primarily in clusters of epithelial cells in the lower third of the crypts; however, we could also find crypts where DCLK1$^+$ cells were scattered among the entire crypt length (Fig. 4C). The epithelial DCLK1$^+$ cells showed a characteristic staining pattern in the cytoplasm. Differentiated cells like goblet cells showed no positivity for DCLK1. We could show a significant upregulation of DCLK1$^+$ cells in Barrett esophagus associated with EAC ($P < 0.0001$), 64% of the 47 EAC specimens had a DCLK1 score of 2/3 compared with 15% of the 65 nondysplastic Barrett esophagus specimens (Fig. 4E), and there was no difference between close (<3 mm) and distant (>3 mm) tissue locations (Supplementary Fig. S1).

Diagnostic model for detecting adjacent EAC

To determine whether the above markers may have utility in the identification of Barrett esophagus harboring occult neoplasia, or if they represent potential markers of high-risk Barrett esophagus, we evaluated how well each biomarker discriminated between the nondysplastic Barrett esophagus and Barrett esophagus with cancer group. Figure 5 depicts the results of the logistic regression models for every biomarker. The ROC curves indicate that TFF2 showed the best discrimination between the Barrett esophagus and EAC group with an AUC of 87.2%, followed by DCLK1 (AUC of 83.4%), goblet cell ratio (AUC of 79.4%), and LGR5 (AUC of 71.4%). The discrimination between the Barrett esophagus and EAC group was improved when combining the various marker proteins. Table 2 shows the results of ROC curves of logistic regression models for all possible marker combinations. The combination of goblet cell ratio + TFF2 + DCLK1 attained an almost perfect discrimination between the EAC and Barrett esophagus group, with an AUC of 99.3% (sensitivity of 1 and specificity of 0.93), closely followed by the combination of all 4 marker proteins, which also revealed an almost perfect discrimination of 98.6%. However, even if information on only 2 of the IHC marker proteins was available, the discrimination was excellent (Table 2).

Discussion

Patients with Barrett esophagus are more likely than the general population to develop EAC, but risk factors that lead to Barrett esophagus progression and molecular biomarkers are not well understood. In a translational study that includes both mouse model data and human tissue samples, we provide evidence to discriminate dysplastic tissue among Barrett esophagus patients based on a low goblet cell ratio, increased LGR5$^+$ cells, increased DCLK1 cells, and decreased TFF2 cells. On the basis of results from the L2-IL1β mouse model for esophageal carcinogenesis, we propose a model in which LGR5$^+$ stem cells from the gastric cardia are recruited into the esophagus where they can differentiate into columnar epithelium, composed of either goblet cells or TFF2$^+$ progenitor cells, cell types that are normally absent from the squamous esophagus.

Our data suggest that the goblet cell ratio, rather than the simple presence of goblet cells, is better at distinguishing nondysplastic Barrett esophagus and Barrett esophagus associated with cancer in humans. Surprisingly, but consistent with previous findings (28, 32), we noted an inverse correlation between the goblet cell ratio and EAC. In combination with IHC for our other biomarkers, we developed a diagnostic model for malignant progression in

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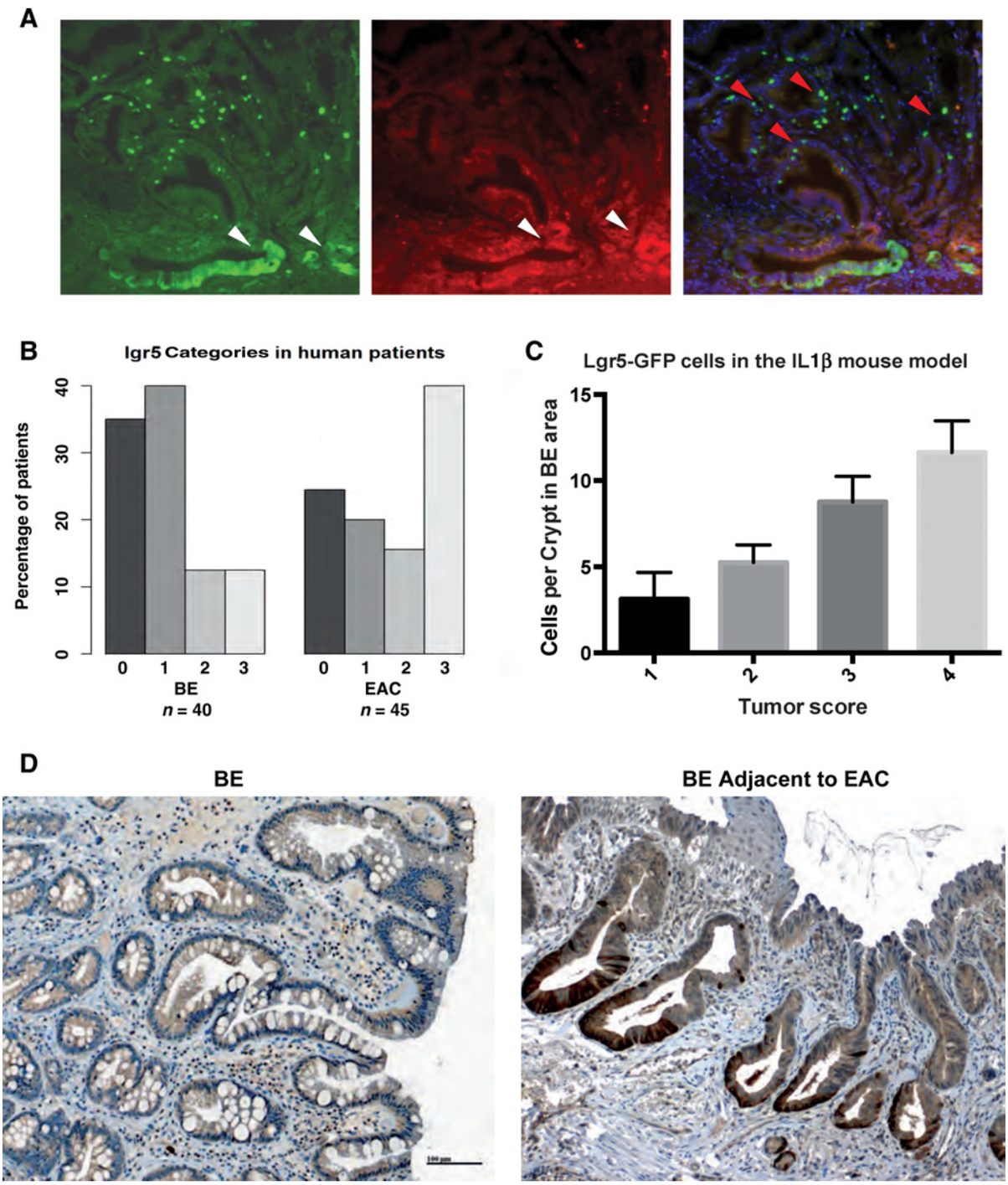

Figure 3.

A, Lineage tracing of Barrett esophagus metaplasia at the SCJ in pL-IL1β mice crossed to Lgr5CreTM mice and Confetti reporter mice at the age of 12 months, 9 months after tamoxifen treatment; white arrows show lineage of red and green clones in Barrett esophagus tissue and red arrows show single Lgr5-GFP–positive cells in Barrett esophagus tissue. **B,** Histopathologic score in correlation with Lgr5-GFP–positive cells at the SCJ in the pL2-IL1β mouse model (ANOVA: $P < 0.0001$, R^2: 0.8418, *F:* 49.65). **C,** Representative LGR5 staining of human biopsies from Barrett esophagus or adjacent to EAC tissue. **D,** LGR5 staining in human biopsies from Barrett esophagus or adjacent to EAC tissue was scored from 0 to 3; percentage of each score in both groups is shown (35%/41%/12%/12% in Barrett esophagus as well as 25%/18%/16%/41% in EAC).

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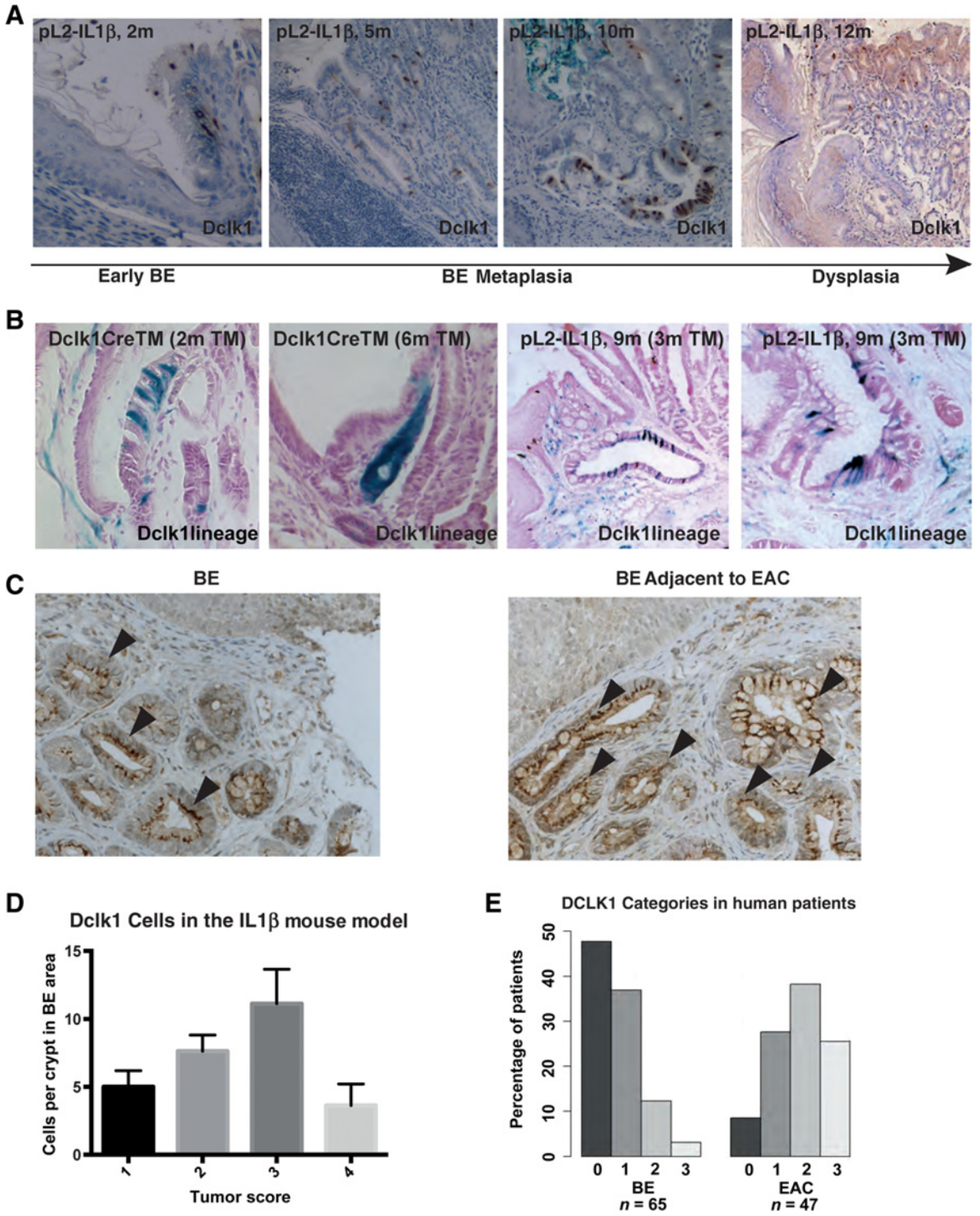

Figure 4.

A, Representative DCLK1 IHC of Barrett esophagus tissue in the pL2-IL1β mouse model with increasing status of early (2 months) to metaplastic (5 and 10 months) Barrett esophagus and dysplasia (12 months). **B,** Lineage tracing of the SCJ of Dclk1CreTM mice crossed to LacZ reporter mice showing DCLK1$^+$ cells at the murine forestomach and a typical lineage tracing event 6 months after tamoxifen administration. In Barrett esophagus metaplasia at the SCJ in pL-IL1β mice crossed to Dclk1CreTM mice and LacZ reporter mice at the age of 9 months, 6 months after tamoxifen treatment, increasing numbers of DCLK1$^+$ cells in Barrett esophagus tissue are shown (ANOVA: $P < 0.0001$, R^2: 0.7600, F: 29.55). **C,** Representative DCLK1 staining of human biopsies from Barrett esophagus or adjacent to EAC tissue; arrowheads pointing at the cytoplasmic location of DCLK1. **D,** Histopathologic score in correlation with Dclk1-CreTm Lac Z–positive cells at the SCJ in the pL2-IL1β mouse model. **E,** DCLK1 staining in human biopsies from Barrett esophagus or adjacent to EAC tissue was scored from 0 to 3, percentage of each score in both groups is shown (48%/37%/12%/3% in Barrett esophagus as well as 9%/28%/38%/26% in EAC).

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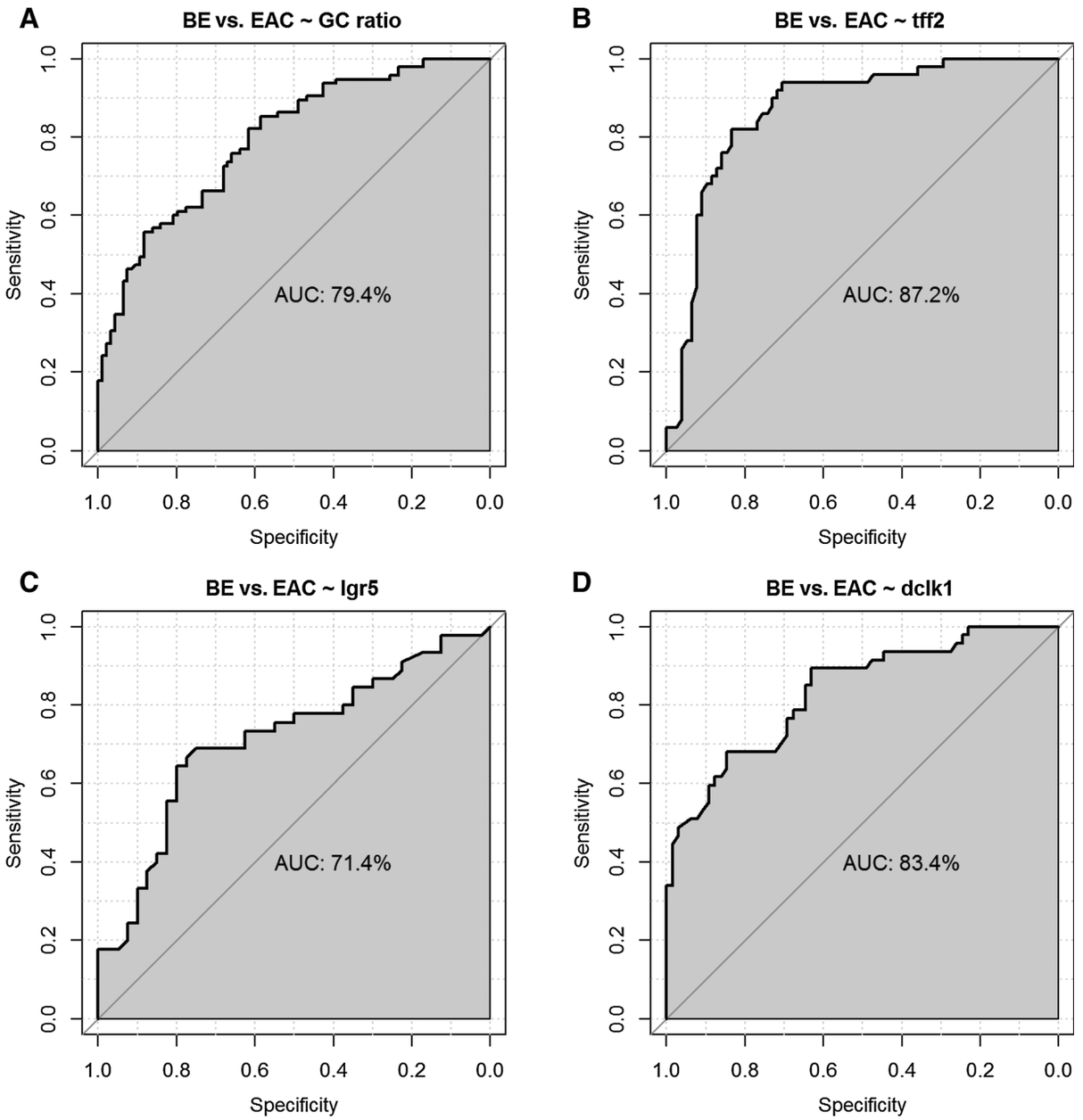

Figure 5.

Logistic regression models for all 4 biomarkers. The ROC plots indicate that TFF2 shows the best discrimination between Barrett esophagus and EAC, with an AUC of 87.2%, followed by DCLK1 (AUC 83.4%), goblet cell ratio (AUC 79.4%), and LGR5 (AUC 71.4%).

Barrett esophagus that can be tested further in prospective trials. Each IHC marker (TFF2, DCKL1, and LGR5) alone showed a strong ability to discriminate nondysplastic Barrett esophagus associated with a patient with early cancer (T1) in an EMR specimen from nondysplastic Barrett esophagus in a patient without any dysplasia in areas of otherwise identical-appearing intestinal metaplasia. Combining these markers in a logistic regression model attained a near-perfect (>98%) discrimination between the Barrett esophagus correlated with EAC and the nonmalignant Barrett esophagus group. Important limitations of these data are the retrospective, single time point nature of the analysis, and thus request the need for confirmation with a predefined cutoff value in independent prospective trials. However, patients with nondysplastic Barrett esophagus that progress to HGD or EAC are quite infrequent, thus making validation of this model a challenging proposition. Of note, our data compare metaplastic tissue in esophagi with or without EAC and therefore represent a field or niche effect typical for malignant transformation that could be secondary to cancer development. Nevertheless, the graduate alterations in the mouse model and the fact that we

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Table 2. Results of ROC curves built on logistic regression models for possible marker combinations

Marker(s) in model	Sensitivity	Specificity	AUC
Goblet cell ratio	0.558	0.883	79.4%
TFF2	0.820	0.833	87.2%
LGR5	0.644	0.800	71.4%
DCLK1	0.681	0.846	83.4%
Goblet cell ratio + TFF2	0.939	0.789	92.5%
Goblet cell ratio + LGR5	0.512	0.923	76.7%
Goblet cell ratio + DCLK1	0.761	0.952	92.7%
TFF2 + LGR5	0.868	0.886	89.0%
TFF2 + DCLK1	1.000	0.814	95.0%
DCLK1 + LGR5	0.903	0.778	89.0%
Goblet cell ratio + TFF2 + LGR5	0.892	0.853	91.3%
Goblet cell ratio + TFF2 + DCLK1	1.000	0.930	99.3%
Goblet cell ratio + LGR5 + DCLK1	0.900	0.923	94.2%
TFF2 + LGR5 + DCLK1	0.963	0.917	96.6%
Goblet cell ratio + TFF2 + LGR5 + DCLK1	0.889	1.000	98.6%

NOTE: All models were adjusted for age and sex. Sensitivity and specificity are obtained by applying the cutoff value that corresponds to the point of the ROC curve for which Youden index reaches its maximum.

only chose early noninvasive T1 cancers in our EMR specimens would argue in favor of a continuous change during carcinogenesis and not a purely secondary effect do to progredient cancer growth. Moreover, a separate analysis taking the distance to the dysplasia in the EAC bearing EMR specimens into account revealed no difference between close and distant tissue, arguing for a broader field effect. Future prospective studies need to evaluate whether these changes can be used to predict the prospective risk of dysplasia development. The definition of a niche of high risk rather than a specific region of dysplastic growth might be important to estimate the malignant potential in patients with even short segment Barrett esophagus or only metaplastic changes at the gastroesophageal junction or cardia.

Our data suggest that goblet cells represent a surrogate for a well-differentiated tissue type that is likely a stable and well-adapted sort of metaplasia, with limited potential for malignant transformation. In many recent gastrointestinal society guidelines (e.g., AGA, BSG, DGVS), the role of goblet cells in Barrett esophagus has been much debated, with most guidelines suggesting that only patients with specialized intestinal metaplasia (i.e., abundant goblet cells) should be diagnosed with Barrett esophagus and included in screening programs for EAC. While intestinal metaplasia is present in the vast majority of Barrett esophagus cases (33, 34), and some goblet cells can almost always be identified when a sufficient number of biopsies was taken, it is clear that EAC can be found in patients without classical Barrett esophagus with intestinal metaplasia, limiting the utility of intestinal metaplasia as the sole risk factor for EAC. Consistent with our surprising findings, a recent study demonstrated that the goblet cell count in Barrett esophagus shows an inverse relationship with the presence of DNA content abnormalities by flow cytometry. This may suggest that a loss of goblet cells, which are terminally differentiated with low proliferative ability, may represent a biologic mechanism necessary for the development of adenocarcinoma (28). Indeed, goblet cells are responsible for secreting components for a mucosal barrier and represent a major cellular component of the innate defense system and might serve mucosa protective in metaplasia (35). We showed a similar inverse correlation with TFF2 protein expression, which has been shown to mark differentiated mucus neck cells in the gastric epithelium, which like goblet cells are a secretory cell type commonly found in Barrett esophagus. Unexpectedly, however, $TFF2^+$ cells are suppressed with progression to EAC, consistent with the loss of other differentiated cell types. TFF2 has been demonstrated to label intestinal metaplasia a long time ago (25, 36) and to be present in Barrett esophagus epithelium in correlation with a secretory phenotype (37) but was never inversely associated with the risk to develop EAC in favor of a more proliferative and less differentiated cell lineage.

In the L2-IL1β mouse model of Barrett esophagus, inhibition of mucus cell differentiation correlates with malignant progression (5). In human Barrett esophagus, columnar-like epithelium seems to precede the development of intestinal metaplasia, and progression to intestinal metaplasia is associated with the extent and the duration of the disease (33). In histopathologic studies of esophageal cancer resection specimens, gastric cardia–type columnar epithelium was found in the esophagus in 100% of cases, but goblet cell metaplasia was found in only 21% (38). In addition, a recent analysis of EAC resection specimen using a combination of histopathologic spatial mapping and clonal ordering demonstrated that EAC developed from a premalignant clonal expansion in columnar metaplasia lacking goblet cells, underlining the premalignant potential of metaplastic columnar epithelium without goblet cells in the context of Barrett esophagus (29). In our analysis, a low ratio of goblet cells in adjacent tissue was significantly associated with the presence of dysplasia or cancer in mouse and human tissue, indicating that a more differentiated intestinal metaplasia tissue might be even protective. Thus, we propose here that the increasing density of goblet cells within Barrett esophagus tissue might be a negative predictor for cancer development in EAC. In addition, one might speculate that a similar marker panel can be used in patients with "carditis" and/or with intestinal metaplasia of the cardia. Indeed, we have hypothesized that gastroesophageal junction cancer and EAC are really the same disease (5, 39) and that EAC may actually originate from gastric cardia stem cells. Our mouse model of Barrett actually begins with carditis and metaplasia of the cardia and does show increased Lgr5 and decreased Tff2 expression during progression to cancer. However, from a clinical perspective, patients with only carditis and lacking clear Barrett metaplasia are typically not well diagnosed placed into surveillance programs, particularly if they show few goblet cells. We would argue that greater attention should be paid to carditis and goblet cell poor metaplasia (e.g., columnar-like epithelium) at the gastroesophageal junction.

The very proximal stomach, or gastric cardia, represents a zone of 4 to 5 gland units just below the SCJ. The murine model of Barrett esophagus and EAC suggests that metaplastic lesions originate from stem cells in the gastric cardia (5, 6, 40), which over time appear to migrate proximally into the squamous esophagus leading to the development of dysplasia. In this study, we show in murine (L2-IL1β) and human models of Barrett esophagus that stem/progenitor cells ($LGR5^+$) are expanded in both the gastric cardia and Barrett esophagus tissue at the SCJ. Using LgR5-Cre-ER^{T2} mice, we were able to demonstrate here that polyclonal metaplasia and dysplasia can arise from a $LGR5^+$ cardia stem cell. This clearly occurs first in the murine cardia, presumably in response to inflammation extending down from the SCJ. As a consequence, multiple clonal populations with highly variable patterns of genomic aberrations may arise (41), and prospective studies have shown that the diversity of clonal

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neoplastic populations arising within the Barrett esophagus segment is a strong, objective predictor of progression to EAC (42). While the presence of LGR5$^+$ cells in Barrett esophagus has been reported previously (43), we have shown here that in Barrett esophagus, an increase in LGR5 expression (a surrogate for undifferentiated cell types) in combination with other differentiation markers seems to predict progression toward EAC. It seems reasonable to assume that EAC arises from such same progenitors that give rise to Barrett esophagus. While some consideration is given to the possibility of "metaplasia of the most distal esophageal squamous epithelium" (44, 45), or submucosal glands in the distal esophagus (44), a proximal extension of original cardiac mucosa is favored by our lineage tracing studies. The finding that Barrett heterogeneity results from multiple independent stem/progenitor clones would argue against a transdifferentiation model. Although origins of Barrett esophagus in humans is still open to debate, recent data from a number of independent groups have demonstrated that Barrett esophagus is more similar histologically to gastric tissue (12). Nevertheless, lineage tracing cannot be performed in humans; therefore, such descriptive studies will for now be the only proof of such a concept in humans.

Dclk1$^+$ marks tuft cells in the gastrointestinal tract, and tuft cells were amplified in regions of metaplasia and predysplasia in the IL1β mouse model as well as in human samples. We recently demonstrated in the colon and intestine that Dclk1$^+$ tuft cells play an important role in modulating the intestinal stem cell niche, as loss of Dclk1$^+$ cells impairs the ability of stem cells to proliferate and expand following injury such as dextran sodium sulfate (DSS) colitis (19). Furthermore, we have previously shown that Dclk1$^+$ cells are expanded in preneoplastic conditions (19), and in Barrett esophagus, Dclk1$^+$ cells were markedly increased in the cardia and metaplastic tissue over time. Taken together, the data suggest that Dclk1$^+$ cells are highly important niche cells supporting the proliferation of stem cells in response to injury or in the setting of carcinogenesis and thus the expansion of Lgr5$^+$ stem cells may occur, in part, due to the expanded niche, although Dclk1$^+$ cells are often decreased with progression to cancer (46, 47). The notion that tuft cells are niche cells responsible for maintaining mucosal homeostasis and communicating to other cell types has recently been demonstrated (48). It is therefore tempting to speculate that tuft cells are important not only during infection but also during carcinogenesis. Here, we show that increasing numbers of these potential niche cells are highly correlated with malignant transformation of the Barrett tissue and could be used as a good predictive biomarker for surveillance, as also proposed by Houchen and colleagues with the expression of DCLK1 in the epithelium, stroma, and plasma of patients with Barrett esophagus/EAC (9).

In conclusion, this translation of mouse biomarkers to a study of patients with Barrett esophagus highlights the importance of preclinical models for the understanding of human disease. The combination of high LGR5 or DCLK1 and low TFF2 or goblet cell ratio into a diagnostic model appears promising in discriminating between nondysplastic Barrett esophagus and Barrett esophagus associated with EAC. The novel integration of differentiation, niche, and stem cell markers into a combined score allows a better discrimination of patients who developed EAC. We utilized Youden index for logistic regression in which all parameters are taken into account to define an ideal score to determine the power of a combined use of distinct markers. Nevertheless, its clinical benefit remains to be determined in further prospective studies. Overall, the data presented here highlight the potential for combining functionally relevant biomarkers, particularly those reflecting a lesser differentiating and stem cell expansion, to address our need for risk prediction tools to better stratify our patients with Barrett esophagus.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R. Schellnegger, M. Quante
Development of methodology: R. Schellnegger, M. Schernhammer, M. Quante
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): R. Schellnegger, M. Schernhammer, B. Höhl, M. Tobiasch, M. Quante
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R. Schellnegger, A. Quante, S. Rospleszcz, M. Schernhammer, M. Tobiasch, A. Brandtner, J.A. Abrams, K. Strauch, M. Vieth, M. Quante
Writing, review, and/or revision of the manuscript: R. Schellnegger, A. Quante, S. Rospleszcz, M. Schernhammer, B. Höhl, A. Pastula, A. Brandtner, J.A. Abrams, K. Strauch, R.M. Schmid, M. Vieth, T.C. Wang, M. Quante
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Schellnegger, M. Schernhammer, M. Tobiasch, A. Brandtner, R.M. Schmid, M. Quante
Study supervision: R. Schellnegger, M. Quante

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Cancer Prevention Research

Goblet Cell Ratio in Combination with Differentiation and Stem Cell Markers in Barrett Esophagus Allow Distinction of Patients with and without Esophageal Adenocarcinoma

Raphael Schellnegger, Anne Quante, Susanne Rospleszcz, et al.

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