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MOLECULAR BIOMARKERS AND ABLATIVE THERAPIES FOR BARRETT'S ESOPHAGUS

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SUMMARY

Barrett's esophagus is the major risk factor for esophageal adenocarcinoma. Endoscopic interventions which ablate Barrett's esophagus mucosa lead to replacement with a new squamous (neosquamous) mucosa, but it can be difficult to achieve complete ablation. Knowing whether cancer is less likely to develop in neosquamous mucosa or residual Barrett's esophagus after ablation is critical for determining the efficacy of treatment. This issue can be informed by assessing biomarkers that are associated with an increased risk of progression to adenocarcinoma. Although there are few post-ablation biomarker studies, evidence suggests that that neosquamous mucosa may have a reduced risk of adenocarcinoma in patients who have been treated for dysplasia or cancer, but some patients who do not have complete eradication of non-dysplastic Barrett's esophagus may still be at risk. Biomarkers could be used to optimize endoscopic surveillance strategies following ablation, but this needs to be assessed by clinical studies and economic modeling.

KEY WORDS:

Barrett's esophagus, esophageal adencarcinoma, ablation, biomarkers

INTRODUCTION

It is well recognized that Barrett's esophagus, a condition where the distal esophageal squamous epithelium is replaced by a metaplastic columnar epithelium, is a complication of chronic gastroesophageal reflux, and is the major risk factor for the development of esophageal adenocarcinoma [1,2]. Esophageal adenocarcinoma is associated with a high mortality rate, and its incidence in the Western world is rising faster than any other cancer, with a six fold increase reported over the last 30 years in both the USA [3,4] and Australia [5]. Conventional treatment for esophageal adenocarcinoma entails esophagectomy. This operation can only be applied selectively to fit patients with localized disease. It is also associated with significant morbidity. In-hospital mortality rates of 13% were reported in a meta-analysis from the 1990's [6], and 8.8% in the 2000's [7]. Undertaking surgery in high volume centers and careful patient selection can reduce mortality to 2-3%, although only 1/3 or less of patients that develop esophageal adenocarcinoma are treated with surgery. Furthermore, the 5-year survival rate following surgery for advanced disease is approximately 22%, and has not improved over the last 20 years [8]. This highlights the need for better approaches to the management or prevention of esophageal adenocarcinoma.

It is generally accepted that Barrett's esophagus progresses from non-dysplastic metaplastic columnar mucosa to low grade dysplasia (LGD), then to high grade dysplasia (HGD), and eventually to invasive adenocarcinoma [9]. It has been estimated that the risk of progression from non-dysplastic Barrett's esophagus to adenocarcinoma for patients enrolled in surveillance programs is approximately 0.5% per patient year [10,11], and in a large North American multicentre study the incidence of esophageal adenocarcinoma was 0.27% per patient year, while the incidence of both esophageal adenocarcinoma and HGD was 0.63% per patient year [12]. In the general population the rate of progression from Barrett's esophagus with intestinal metaplasia to high grade dysplasia and esophageal cancer has been estimated at 0.38% per year in Northern Ireland [13], and the progression rate to cancer for columnar lined esophagus (with or without intestinal metaplasia) at 0.12% per year in Denmark [14].

The recognizable stages of progression in Barrett's esophagus provide opportunities for the use of endoscopy to identify patients at earlier and more curable disease stages; i.e. HGD or early (T1 stage) adenocarcinoma. It also provides opportunities for preventative strategies which might reverse the steps towards cancer, or prevent cancer progression. These all have the potential to

greatly improve clinical outcomes. Five year survival rates following surgery for T1 stage cancer of approximately 80% highlight the potential for improvement if interventions can be undertaken early.

To reverse the process of Barrett's esophagus to a normal (neo-squamous) epithelium various interventions have been proposed. Initially some physicians advocated regular use of proton pump inhibitors (PPIs), whereas some surgeons advocated antireflux surgery [15-18]. However, whilst isolated case reports have suggested regression of Barrett's esophagus following antireflux surgery, the rates of regression have been disappointing following both medical and surgical treatment of reflux [15,16]. Indeed, there are multiple reports of esophageal adenocarcinoma developing after antireflux surgery [17,19-21], and many patients presenting with advanced stage esophageal cancer report long periods of PPI use.

Endoscopic ablation

Newer interventions which destroy (ablate) the metaplastic columnar lining associated with Barrett's esophagus are potentially more effective than PPIs or anti-reflux surgery. It appears that any method which removes the metaplastic mucosa, whether or not it is dysplastic, in the presence of an acid-free environment, will lead to replacement with a neosquamous mucosa. This mucosa is histopathologically similar to normal squamous epithelium, and it has therefore been assumed to have a reduced risk of cancer [22,23]. A variety of different ablation techniques have been developed, including argon plasma coagulation (APC) [24-27], photodynamic therapy (PDT) [28-30], multipolar electrocoagulation (MPEC) [31,32], Nd:YAG (neodymium-doped yttrium aluminum garnet) laser therapy [33-35], and more recently radiofrequency ablation (RFA) [36,37]. The optimum technique has yet to be defined, but the use of these techniques, especially RFA, is becoming more widespread [38]. All ablation modalities, however, are associated with a risk of residual or recurrent Barrett's esophagus, with no single technique 100% reliable in this regard (Table 1) .

In a randomized trial, Hage et al [39] compared PDT and APC and found a 33% rate of recurrence of Barrett's esophagus following APC at 12 months. PDT was followed by recurrence in 18% when administered as a single dose, or 10% when delivered as a fractionated dose. Conversely, Kelty et al [40] in a randomized trial comparing PDT and APC found complete reversal of Barrett's esophagus in 97% of APC patients but only 50% of PDT patients at median 12 months follow up. In a prospective study of APC ablation, Van Laethem et al re-evaluated 17 patients, who had complete

ablation of their Barrett's esophagus, 12 months after ablation treatment, and found recurrent Barrett's esophagus in 8 (47%) [24]. Studies investigating MPEC have been comparable. Sharma et al [32] compared MPEC with APC in a randomized trial and reviewed the outcomes at 2 years. Complete reversal of Barrett's esophagus was seen in 75% (12/16) of the patients who received MPEC, and 63% (12/19) of the patients who received APC. In a prospective evaluation of 58 patients with nondysplastic Barrett's esophagus treated with MPEC, 22% had residual Barrett's esophagus at 6 months follow up [33]. In a randomized trial of APC versus surveillance for non-dysplastic Barrett esophagus after antireflux surgery, only 40% of patients had complete reversal of Barrett's esophagus at 5 years [27]. In the only long term follow up study after PDT, Overholt et al (2007) reported that 48% of patients had complete reversal of HGD after 5 years, but no data was given for complete reversal of Barrett's esophagus. Furthermore, at 5 years 15% of the PDT ablated patients had developed cancer, compared with 30% taking omeprazole [41].

Laser ablation of Barrett's esophagus is also associated with a significant risk of residual Barrett's esophagus. In a prospective study of Nd:YAG laser ablation of non-dysplastic Barrett's esophagus [34] followed by treatment with PPIs or antireflux surgery, Bonavina et al (1999) achieved only partial ablation in 28% (5/18) of patients despite repeated treatments, and 11% (2/18) were defined as "non-responders". In another prospective study Nd:YAG laser was used to ablate Barrett's esophagus in 15 patients (11 non-dysplastic, 2 with LGD and 2 with HGD) [35]. Complete endoscopic and histopathological ablation was only achieved in 6 (40%) patients after a mean of 6.5 laser treatment sessions.

Fleischer et al (2010) reported outcomes for RFA of nondysplastic Barrett's esophagus after 5 years. Complete reversal of Barrett's esophagus was demonstrated in 92 % (46/50) of patients, and focal RFA ablated the remaining Barrett's esophagus in the remaining 8% of patients [42]. Despite these encouraging results, the rate of complete remission of Barrett's esophagus following RFA for dysplasia or cancer appears to be an issue. Shaheen et al reported that at 3 years dysplasia remained eradicated in >85% of patients and intestinal metaplasia in >75% [43], but this data also revealed a persistent risk of cancer in some patients, although perhaps the overall risk of cancer was reduced across the whole cohort. Lyday et al (2010) also found complete remission of non-dysplastic Barrett's esophagus in 77% of patients (n = 137) at 20 months follow up [36], and Ganz et al (2008) found a 54% complete remission rate for non-dysplastic Barrett's esophagus at median 12 months follow up [37].

Another concern relating to the use of ablative therapies is the incidence of residual columnar mucosa buried beneath post-ablation neosquamous mucosa ("buried glands"). This has been reported to occur in up to 40% following PDT ablation [29,30], and in 30% of patients treated with APC [24,27,44]. It might be less of an issue following RFA ablation, with only 2 case reports of buried glands published [45,46], although this technique is more recent and less outcome studies are available. Table 2 summarizes publications that have demonstrated buried glands following different ablation modalities. There are now multiple case reports of adenocarcinoma arising in buried glands beneath neosquamous mucosa following ablation[34,47-50], suggesting that this might be a significant problem. However, the overall risk profile of residual or recurrent Barrett's esophagus and buried glands following ablation is uncertain, and it is also possible that buried mucosa, whilst still at risk of progression to cancer, could have a reduced risk of cancer. To address the issue of cancer risk in residual Barrett's esophagus and neosquamous mucosa, long term clinical outcome studies involving large patient cohorts are required. These are unlikely to be reported in the next decade. Hence, molecular biology studies which evaluate gene function in esophageal mucosa following ablation therapy, i.e. biomarker studies, might provide insights into the likely behavior of post-ablation esophageal mucosa, and the risk of progression to cancer following ablation. Knowing whether cancer development in Barrett's esophagus is less likely or even eliminated by ablation is important. In the absence of evidence that cancer risk is reduced to a very low level, ablation therapy might not be regarded as clinically effective or cost effective by health care decision makers.

To evaluate the current biomarker studies pertinent to these questions, we performed a Medline search on 14 December 2012, and then updated this search on 15 February 2012. Relevant publications dealing directly with biomarkers, esophageal cancer, and ablative therapies were obtained by screening the abstracts or, if necessary, the entire article. Further articles were extracted by screening the references within these papers. In case of non-availability of the whole article the abstract was taken into consideration despite the limited data provided. No publications were found that investigated biomarkers in patients treated with endoscopic mucosal resection.

Molecular biomarkers in Barrett's esophagus

A large number of molecular markers have been identified and proposed to be relevant to Barrett's esophagus, but for most there is limited data which supports either relevance or a clinically useful role. For only a limited number of biomarkers does the data show relevance to the behavior and

cancer risk associated with Barrett's esophagus. The most promising molecular biomarkers in Barrett's esophagus are summarized.

p53

p53 is a tumor suppressor gene located on chromosome 17p. Its role is to prevent damaged cells from dividing and to ensure chromosomal integrity [51]. Its function is impaired either by inactivation (loss of function) mutations that prevents the protein from binding to DNA, by dominant negative mutations that result in the prevention of transcriptional activation [52,53], or by gain-of-function mutations that are involved in aberrant protein interactions or gene regulation [54]. p53 can also be inactivated through 17p loss of heterozygosity (LOH), and mis-sense mutations appear to often result in p53 protein over-expression [55]. Mutation of p53, 17p LOH and over-expression of p53 protein are rarely detected in normal non-metaplastic esophageal mucosa, but are common in esophageal adenocarcinoma [56,57].

Weston et al looked at p53 protein expression in Barrett's esophagus [58] in 48 patients with LGD, followed for a mean 41 months. Five progressed to HGD and 3 of these had Barrett's esophagus mucosa which over-expressed p53 protein. Twelve had persistent LGD and 3 over-expressed p53 protein. Thirty one regressed to non-dysplastic Barrett's esophagus, and in this group, 4 over-expressed p53 protein. Kaplan-Meier survival curves were performed using progression to HGD or cancer as an endpoint vs. regression or persistence of LGD, and a significant difference ($p < 0.002$) was seen, leading to the conclusion that over-expression of p53 protein identified patients with LGD who were at higher risk of progressing to HGD. Unfortunately, however, although over-expression of p53 protein is frequently considered to be a surrogate marker for p53 mutations, it has been shown to have a high false-negative and false-positive (>25%) rate in esophageal cancer, compared to DNA sequencing [59]. Furthermore, even though Murray et al (2006) showed immunohistochemical detection of p53 protein over-expression is associated with an increased risk of progression to cancer, the sensitivity of this marker was too low to be useful for informing endoscopic surveillance strategies [60].

Reid et al (2001) published the only large study looking at p53 and Barrett's esophagus [61]. In this study 256 patients had baseline endoscopic biopsies from Barrett's esophagus mucosa and one or more follow up endoscopies. Twenty out of 54 patients (37%) with p53 LOH progressed to cancer compared with 6 of 202 patients (3%) without (relative risk (RR) = 16; $p < 0.001$). The 3-yr

cumulative incidence of cancer in patients with 17p (p53) LOH was 38% (95% CI = 26 - 54) compared to 3.3% (95% CI = 1.4 - 8.0) for those with two 17p alleles. Patients without dysplasia had a p53 LOH prevalence of 6%, while this increased to 57% in patients with HGD. This study suggests that p53 LOH remains the most promising molecular biomarker for the prediction of progression of Barrett's esophagus to cancer.

In a follow-up study from the same group, Galipeau (2007) investigated 17p (p53) LOH, 9p (p16) LOH, aneuploidy, and tetraploidy in 253 patients with Barrett's esophagus, and reported cumulative cancer incidences at up to 10 years. At 10 years 17p LOH gave a relative risk of 10.6 (95% CI 5.2 - 21.3). The combined panel of abnormalities (17p LOH, DNA content tetraploidy and aneuploidy, and 9p LOH) was the best predictor of esophageal adenocarcinoma, with a relative risk of 38.7 (95% CI 10.8 - 139) [62].

However, LOH genotyping is currently limited to the research setting. If an alternative method of assessing LOH, such as fluorescence in situ hybridization (FISH), could be shown to provide equivalent results, then translation to clinical practice might be feasible. However, LOH develops in Barrett's esophagus by three mechanisms. In a study by Wongsurawat et al (2006), 32% of patients had DNA deletions, 32% had no copy number change, and 37% had FISH patterns consistent with a tetraploid intermediate followed by genetic loss. Thus, FISH and LOH are not equivalent [63].

DNA content (aneuploidy/tetraploidy)

Both aneuploidy and tetraploidy are rare in normal tissue, but common in esophageal adenocarcinoma [64]. In the largest study to assess this in Barrett's esophagus, the Seattle Barrett's Esophagus Study group prospectively evaluated 327 patients over 15-years [65]. Median follow-up was 2.4 years. In patients with nondysplastic Barrett's esophagus and LGD, those without aneuploidy or tetraploidy had a 0% 5 year cumulative cancer incidence, whereas in patients with aneuploidy or tetraploidy the 5-year cumulative cancer incidence was 28%. For all patients, either with and without HGD, aneuploidy and tetraploidy detected at baseline were associated with a 43% and 56% 5 year cancer incidence respectively. Of the 327 patients studied , 322 had baseline histopathology, flow cytometry dat,a and matched data from at least one follow up endoscopy. 241 (75%) had neither aneuploidy nor tetraploidy at baseline, and only 12 (5%) of these progressed to cancer, whereas 48 had tetraploidy at baseline with 18 (38%) progressing to cancer. Tetraploidy was associated with a relative risk of cancer of 7.5 (95% CI = 4.0 - 14)($p < 0.001$). Fifty three

patients had baseline aneuploidy and 17 (32%) developed cancer, which equated to a relative risk of 5 (95% CI = 2.7 - 9.4; $p < 0.001$). The relative risk when looking at aneuploidy, tetraploidy or both was 11 (95% CI = 5.5 - 21; $p < 0.001$). The follow up paper from this group [66] reported receiver operating characteristic (ROC) curve analysis to better define the relevant cutoff points of DNA flow cytometry analysis. Patients whose biopsies contained 6% or more tetraploid cells had a relative risk of 11.7 of developing cancer. Aneuploidy greater than 2.7N was also predictive of cancer with a relative risk of 9.5. The presence of both tetraploidy greater than 6% and aneuploidy greater than 2.7N was associated with a relative risk of 23 and was highly predictive [65].

In another study looking at DNA content and Barrett's esophagus [67], the Seattle Barrett's Esophagus study group prospectively analyzed a different cohort of patients, and determined at baseline in 267 patients the approximate size of the clone (i.e. the number of cells that are genetically unstable) with aneuploidy and tetraploidy, rather than just the presence of ploidy abnormalities. The size of the clone was defined as a product of the length of Barrett's esophagus (cm) and the fraction of cells in the biopsies that carried aneuploidy and tetraploidy. Esophageal adenocarcinoma was the end point. Patients were followed prospectively for a mean of 4.4 years. The size of a clone with ploidy abnormalities had a relative risk of 1.31(x) (for an x cm clone; 95% CI, 1.07-1.60) in predicting progression to esophageal adenocarcinoma. Controlling for length of the Barrett's esophagus segment had little effect. This was a significantly better predictor of progression than just the presence of these clones. These studies confirm that DNA content remains a strong candidate as a marker of progression to esophageal adenocarcinoma.

p16

p16 is a tumor suppressor gene that plays a key role in regulating the cell cycle. In particular, it controls the transition from G1 to S phase [68]. p16 can be inactivated through mutation, LOH or methylation. Wong et al (2001) observed that the mucosa from more than 85% of Barrett's esophagus segments contains tissue in which at least one p16 gene is inactivated [69], and at least one of the p16 lesions (p16+/- or p16-/-) demonstrated extensive clonal expansion. There was a significant association between the severity of the p16 lesion (p16+/+ vs. p16+/- vs. p16-/-) and the length of Barrett's esophagus (median 1.5 to 6.0 to 8.0 cm). This led to the conclusion that p16 lesions facilitate clonal expansion, which then provides an environment where further genetic abnormalities are more likely to arise. In addition, the grade of dysplasia did not influence the prevalence of the p16 lesions. This led the authors to also conclude that inactivation of p16 occurs early in the progression of Barrett's esophagus, a finding that has been supported by Bian et al

(2002) [70]. Despite this early promise, however, there are no prospective studies which have demonstrated that p16 lesions can predict progression to cancer. In the largest prospective analysis, Maley et al (2004) showed that the ability of the size of a clone containing a p16 lesion to predict cancer was lost when it was corrected for the presence of p53 LOH [67].

Cyclin D1

Cyclin D1 is a cell cycle protein that promotes the transition from G1 to S phase. Cyclin D1 acts as an antagonist to p16, and together they regulate the cell cycle. When p16 is present cyclin D1 is prevented from promoting cell division [71]. In a prospective endoscopic surveillance case-control study, Bani-Hani et al (2000) [72] demonstrated that patients with cyclin D1 over-expression in Barrett's esophagus were more likely to develop esophageal adenocarcinoma, than those without (OR = 6.85; 95% CI, 1.57-29.9; $P < 0.01$). This study compared 12 patients who developed esophageal adenocarcinoma with 49 matched controls with Barrett's esophagus. The results suggested that cyclin D1 over-expression was associated with a 6 to 7x increased risk of esophageal adenocarcinoma. However, this study did not find a link between p53 and increased risk (OR = 2.99; 95% CI = 0.57-15.76). In contrast, another case control study compared 35 patients with Barrett's esophagus who progressed to either esophageal adenocarcinoma or HGD [60] with 163 controls matched for age, sex and date of diagnosis of Barrett's esophagus. With mean follow up of 3.7 years the authors were unable to establish a relationship between cyclin D1 over-expression and cancer risk.

Proliferation abnormalities

Barrett's esophagus is known to be hyperproliferative [73]. Studies looking at proliferation suffer, however, because of the absence of a standardized assay and definition. Proliferation can be measured either on the luminal surface or across the width of the Barrett's esophagus mucosa. One of the more common markers of cellular proliferation is the protein Ki-67. It is present during active phases of the cell cycle but absent during quiescence [74]. Chao et al (2008) [75] used Ki-67 and flow cytometry to measure total proliferation. They prospectively followed 276 patients with Barrett's esophagus for a mean of 6.3 years. Twenty nine developed esophageal adenocarcinoma. Ki67 positivity ($p = 0.13$) and G1 fractions ($p = 0.15$) as measured by flow cytometry were not associated with progression to cancer.

Sirieix et al (2003) [76] used minichromosome maintenance protein 2 (Mcm2) as a luminal proliferation marker in a case control study. Minichromosome maintenance proteins are expressed during the cell cycle and are then degraded once cells become differentiated [77]. In this study 9 cases of esophageal adenocarcinoma had greater Mcm2 expression before the diagnosis of dysplasia compared with matched controls (28.4% of total surface epithelial cells vs. 3.4% in the control group, $p < 0.0001$). No further work has been done on validating Mcm2 as a marker of esophageal adenocarcinoma risk.

The Seattle Barrett's Esophagus study group studies also assessed proliferation as a marker for cancer progression [66,77]. Rabinovitch et al (2001) [66] reported the use of flow cytometry to measure the S phase of the cell cycle as a marker of proliferation. ROC curve analysis suggested optimal cut-off points of greater than 5.5% and 9% at baseline endoscopy. Out of 307 patients, 137 (45%) demonstrated an S-phase fraction greater than 5.5%, and 44 (14%) had an S-phase fraction greater than 9%. The incidence of cancer at 3 years in the first group was 17% (CI = 12-25) and at 5 years was 21% (CI = 15-30). On univariate analysis there was a relative risk of 2.3 (CI = 1.2-4.4, $p = 0.02$) in patients with an elevated S phase fraction . The incidence of cancer at 3 years in the group with an S-phase fraction greater than 9% was 21% at 3 years (CI = 11-40) and 28% at 5 years (CI = 14-51). The relative risk for this group was 2.0 (CI = 0.94 – 4.1, $p < 0.07$). All these risks, however, lost significance in multivariate analysis when looking at patients with and without HGD. In both groups the relative risk of an S-phase fraction greater than 9% was 1.0. Based on these studies there is little evidence to support using proliferation as a marker for progression of Barrett's esophagus to adenocarcinoma.

Promoter methylation

The epigenetic addition of methyl groups to DNA at CpG islands has been established as a common mechanism of gene inactivation in carcinogenesis [78,79]. It is well accepted that promoter hyper-methylation is a mechanism of tumor suppressor gene silencing. Hyper-methylation has been observed in primary Barrett's esophagus and esophageal adenocarcinoma tissues, and is the most frequent mechanism of APC and p16 inactivation in esophageal adenocarcinoma [80,81]. In a study in Barrett's esophagus, Schulmann et al (2005) [82] investigated promoter methylation of 10 genes. Based on a dichotomized categorization, 3 genes (HPP1, RUNX3, and p16) were methylated more frequently in esophageal adenocarcinoma than in Barrett's esophagus. In a longitudinal validation study, multivariate analyses suggested that hypermethylation of these 3 genes were independently

associated with an increased risk of progression: p16 (OR 1.74, 95% CI 1.33–2.20), RUNX3 (OR 1.80, 95% CI 1.08–2.81), and HPP1 (OR 1.77, 95% CI 1.06–2.81). In combined analyses, risk was detectable up to, but not earlier than, 2 years before the development of cancer or HGD. In a study of tissues microdissected from formalin-fixed paraffin-embedded sections, APC, TIMP3, and TERT promoters were hypermethylated in 100%, 91%, and 92% respectively in 12 Barrett's esophagus cases that progressed to adenocarcinoma, whereas methylation of these 3 genes was found in only 36%, 23%, and 17% in 16 patients with Barrett's esophagus who did not progress [83].

In a multicenter, double-blinded validation study Jin et al (2009) evaluated a linear combination of eight methylation biomarkers (p16, RUNX3, HPP1, NELL1, TAC1, SST, AKAP12, and CDH13) using coefficients from a multivariate logistic regression analysis, and developed a risk stratification strategy to predict neoplastic progression in Barrett's esophagus, based on the eight markers. At high specificity levels, this model predicted approximately half of the HGDs and esophageal adenocarcinomas that would not have otherwise been predicted [84]. The eight-marker panel appeared to be more quantifiable, and possess higher predictive sensitivity and specificity than conventional clinical and demographic features. This work, including an additional 55 methylation markers, and a method for determining the risk of disease progression in the context of regular surveillance endoscopy, has been patented (patents WO 2009/105533 and PCT/US/2009/034508).

Brock et al (2006) reported that positive methylation status for multiple genes in esophageal adenocarcinoma was a predictor of poor prognosis [85], and concluded that profiling by methylation status was a more powerful predictor of risk than clinicopathological features of stage and age. These authors also suggested that the frequency of DNA methylation events reflects disease progression, and that DNA methylation events accumulate as cancer advances with time. More recently Kaz et al (2011) [86] used methylation microarrays to measure global gene methylation status in biopsies from patients and found distinct global methylation signatures, as well as differential methylation of specific genes, that discriminated between squamous mucosa, Barrett's esophagus, HGD, and esophageal adenocarcinoma. However, these authors concluded that additional validation of the methylation markers that distinguished non-dysplastic Barrett's esophagus from HGD and cancer is needed before this approach can be applied clinically.

Based on the results of current studies, p53 inactivation, the presence of aneuploidy/tetraploidy, and gene-specific promoter methylation remain the most promising molecular biomarkers for

identifying and predicting progression of Barrett's esophagus to adenocarcinoma. The significant studies which support this conclusion are summarized in Table 3.

Molecular biomarkers and ablative therapies

The application of molecular biomarkers has potential to predict the response of Barrett's esophagus to ablation therapy. In particular, these markers might be able to inform the risk of cancer arising in the 3 different types of epithelium that can be present following ablation: recurrent or residual Barrett's esophagus, neo-squamous epithelium, and "buried Barrett's glands". A number of studies have investigated this area. Although the number of patients in each study has been relatively small, and some of the biomarkers that have been used have only shown limited evidence of association with the risk of disease progression (see Table 4), there is sufficient evidence to draw conclusions that can inform clinical practice.

Recurrent or residual Barrett's esophagus

Histology vs Biomarkers – case evidence

In the earliest study to investigate the effect of ablation on genetic abnormalities in Barrett's esophagus mucosa, Krishnadath et al (2000) [87] looked at archived biopsies from 3 patients who initially responded to PDT but subsequently developed HGD. Proliferation, aneuploidy, p53 protein over-expression, p53 mutations and p16 methylation were evaluated. In all 3 patients histopathological improvement was initially demonstrated, before HGD developed later. All 3 patients had at least 1 or more persistent abnormal biomarkers after PDT. This was the first study to demonstrate persistent genetic abnormalities, despite phenotypic improvement after ablation, suggesting that histopathological improvement may not be an appropriate measure of outcome.

Evidence for complete ablation of Barrett's esophagus

In 2005 Hage et al (2005) [88] looked at the effect of APC and PDT ablation on p53 protein over-expression, proliferation measured by Ki-67, and DNA ploidy status in residual or recurrent Barrett's esophagus mucosa. They evaluated tissue from 29 patients, 16 with nondysplastic Barrett's esophagus, 5 with LGD and 8 with HGD. Patients were followed using endoscopy and biopsy at 1 month, then 3 monthly intervals to 1 year, and 6 monthly thereafter. Mean follow up was 20 months. At 1 month 9 patients (32%) had a complete endoscopic and histopathological regression with significant improvement in both ploidy status and the degree of proliferation.

Patients with residual areas of Barrett's esophagus at 1 month were retreated with APC, resulting in resolution of Barrett's esophagus in 75%. This left 7 patients with persistent Barrett's esophagus - 5 nondysplastic, 1 LGD and 1 HGD. The patient with HGD had persistent p53 protein over-expression and abnormal ploidy status. Persistent abnormal proliferation was seen in 2 of the patients with nondysplastic Barrett's esophagus, and in the patient with LGD. The authors concluded that persistent Barrett's esophagus is still at risk of progressing to esophageal adenocarcinoma. However, the inconsistency of evidence supporting proliferation as a marker of risk raises questions about this conclusion. In a subsequent study Hage et al (2006) [89] undertook LOH analysis on 9 polymorphic markers, including p16 and p53, before and at mean 20 months after ablation. In 5 patients with persistent Barrett's esophagus, the frequency of LOH of p53 and p16 (as well as the APC, DCC and SMAD4 genes) in the residual or recurrent Barrett's esophagus was not decreased after ablation. The authors concluded that the goal of ablation therapy must be complete ablation.

In another study looking at molecular biomarkers in residual Barrett's esophagus [90] Hornick et al (2008) evaluated non-buried Barrett's esophagus mucosa before vs. after ablation in 12 patients with HGD or intramucosal adenocarcinoma, treated with PDT. All had residual or recurrent nondysplastic Barrett's esophagus at follow up. The markers assessed included Ki67, p53, cyclin D1 and DNA ploidy status. Pre ablation biopsies revealed elevated Ki67 proliferation in 43%, p53 positive staining in 8%, but only mild aneuploidy in 73% of cases. Cyclin D1 was absent in all preablation biopsies. The rate of Ki67 proliferation remained unchanged, but they observed a decrease in the number of patients with aneuploidy, to 11% of cases. Although a decrease in the number of patients with mild aneuploidy was observed after ablation, this study still suggests that some patients with residual Barrett's esophagus may be at risk of cancerprogression.

Biomarker evidence for negative effect of ablation on residual Barrett's esophagus

Dvorak et al (2006) [91] compared esophageal mucosal biopsies before and after ablation with MPEC and APC from 21 patients with non-dysplastic Barrett's esophagus. Pre-ablation biopsies revealed normal staining patterns for p53, cyclooxygenase-2 (COX-2) in interstitial cells, and Ki67. Postablation biopsies, however, found increased staining for p53, COX-2 and Ki67 in columnar epithelium located at the junction of the neosquamous and residual Barrett's esophagus in patients whose Barrett's esophagus was not completely ablated. Thirteen of 21 (67%) had increased Ki67 staining at this junction. Eight of 21 (38%) had increased expression of COX-2 and 8 of 21 (38%) had increased staining of p53. The authors concluded that ablation might actually be converting

nondysplastic Barrett's esophagus to areas of dysplastic Barrett's esophagus with more genetic abnormalities within the areas of residual columnar mucosa. They suggested close follow up of all patients following ablation. However, this conclusion was not supported by changes in histopathology. Nevertheless, it does provides some support for the hypothesis that areas of residual Barrett's esophagus after ablation may be at risk of progression to cancer, and provides further evidence that incomplete ablation of Barrett's esophagus may carry some risk of cancer development.

Clinical evidence after Radiofrequency Ablation

Although there have been no biomarker studies yet reported which evaluate residual areas of Barrett's esophagus after radio frequency ablation (RFA), a recent report of 5 year follow up of a prospective multicenter US trial showed complete remission of Barrett's esophagus in 92% (46/50) of patients [42]. Furthermore, Vaccaro et al (2011) investigated forty-seven patients who underwent RFA and had complete eradication of Barrett's esophagus epithelium, and the cumulative incidence of newly detected Barrett's esophagus at one year was 25.9%. Importantly, these authors detected dysplasia at the time of recurrence in four patients. Despite the lack of direct biomarker evidence, these studies suggest that RFA may not eliminate the risk of progression to cancer in all patients [92], an outcome consistent with clinical data from a randomized trial of RFA ablation vs. endoscopic surveillance which suggested a reduced short term risk of progression to cancer, but with a proportion of patients still progressing to cancer within 12 months of RFA ablation [93].

Neo-squamous epithelium

More evidence for complete ablation of Barrett's esophagus

The first study looking at molecular biomarkers in neosquamous epithelium was published by Garewal et al in 1999 [94]. This study involved 2 groups. The first group consisted of 11 patients with Barrett's esophagus (7 nondysplastic and 4 LGD) in whom complete reversal of the Barrett's esophagus had been achieved using a combination of PPIs and MPEC. The second group involved 14 patients in whom ablation was not complete, and islands of squamous mucosa were seen in the Barrett's esophagus mucosa. Control biopsies were taken from normal squamous epithelium in the proximal esophagus as well as from patients without Barrett's esophagus who underwent endoscopy for other reasons. Ki67 proliferation, p53 protein over-expression and ornithine decarboxylase (ODC) activity were assessed in the mucosal biopsies. In the first group Ki67, p53 and ODC measurements were indistinguishable in post-ablation neosquamous epithelium vs.

normal squamous epithelium from controls, suggesting that these patients may be at low risk of subsequently developing cancer following complete ablation. In the second group (squamous islands), however, in contrast to normal squamous epithelium where Ki-67 staining was detected in the basal layer only, multi-layer staining was observed in nine of the 14 cases (64%) of squamous islands, and positive p53 staining was present in 43% (6/14), again highlighting the importance of complete ablation

Neosquamous epithelium may be at risk in some patients

In a subsequent study, Lopes et al (2005) [95] evaluated p53 expression in Barrett's esophagus, squamous epithelium contiguous with Barrett's esophagus, and neo-squamous epithelium before and after APC ablation. Mucosa from 5 of 37 (13.5%) patients over-expressed p53 before ablation, and in each of these 5 patients the squamous mucosa contiguous with Barrett's esophagus also over-expressed p53. In the 32 patients that did not over-express p53, the contiguous squamous mucosa was also found to be negative. Following ablation, the neosquamous epithelium continued to over-express p53 in the 5 cases where this was present beforehand, and in the other 32 cases p53 was not over-expressed in the neosquamous epithelium. This study suggested that despite adequate ablation, post-APC ablation neosquamous epithelium may still have neoplastic potential.

Origin of neosquamous epithelium

Paulson et al (2006) [96] attempted to analyze the origins of neosquamous epithelium developing in patients taking PPI medication, but not undergoing ablation. If neosquamous epithelium originates from the same multipotent progenitor cells that give rise to Barrett's esophagus, then it should share the same genetic profile and subsequent cancer risk. Twenty patients with Barrett's esophagus were identified in whom, either a p16 (9 patients) or p53 (11 patients) mutation within a clonal expansion of Barrett's esophagus cells was evident. All of these patients had islands of neosquamous epithelium confirmed endoscopically and histopathologically. In only 1 patient did the neosquamous epithelium contain the identical p16 mutation that was found in the surrounding Barrett's esophagus. This suggested that in most cases (95%) the neosquamous epithelium and Barrett's esophagus remain genetically distinct, although a small proportion do share a progenitor cell, and maybe the associated cancer risk, a suggestion that is supported by the results of a recent investigation into stem cell organisation in esophageal squamous epithelium and in Barrett's esophagus [97]

Need for appropriate comparison tissues

In another study investigating p53 protein over-expression, 12 patients with HGD underwent PDT ablation [98]. There was significantly lower p53 immunostaining in the neosquamous epithelium from patients who had undergone ablation compared with 10 controls (HGD with no ablation). These authors concluded that the cancer risk of the neosquamous epithelium might be less than that of any HGD that remains after ablation. However, they did not compare neosquamous epithelium with matched proximal squamous tissue, or with normal squamous tissue from healthy patients, so normalization of the gene expression pattern was not adequately assessed.

Evidence for normalization of neosquamous epithelium by Radiofrequency Ablation

The first study looking at biomarkers in neosquamous epithelium following RFA was published by Pouw et al in 2009 [99]. Twenty-two patients with Barrett's esophagus containing either HGD or intramucosal adenocarcinoma underwent RFA. An aggressive approach to ablation was applied with endoscopic mucosal resection undertaken to remove any areas of residual Barrett's esophagus persisting after 5 RFA treatments. Untreated proximal esophageal squamous epithelium was used as a control. Baseline Barrett's esophagus and postablation neosquamous epithelium were assessed for Ki67 and p53 protein expression. Numerical chromosomal abnormalities were evaluated using fluorescent in situ hybridization (FISH) with centromeric enumeration probes for chromosomes 1 and 9, and locus specific identifier probes for regions of 9p21 (p16) and 17p13.1 (p53). All baseline Barrett's esophagus mucosal specimens revealed immuno-histochemical staining and FISH abnormalities in the Barrett's esophagus. All post RFA ablation neosquamous mucosal samples were reported to be normal [99].

Recently Krishnan et al (2012) reported that RFA reduces ß-catenin activity of previously dysplastic mucosa within the regenerative basal epithelial layer to normal levels [100]. This is a potentially important observation as there are several studies implicating ß-catenin signaling in Barrett's esophagus carcinogenesis [101-103], and nuclear ß-catenin appears to be a good marker of dysplasia and esophageal adenocarcinoma [104]. Given the results of Garewal et al (1999), and the fact that these post-RFA biomarker studies were undertaken at a time when all Barrett's esophagus was eradicated, but presumably before the re-emergence or re-establishment of areas of recurrent Barrett's esophagus (e.g. 9% IM at 5 years by Fleischer et al 2010), it appears that complete removal of all Barrett's esophagus is probably required for molecular normalization of the entirety of the esophageal epithelium following ablation.

Buried glands

After proton pump inhibitor therapy

Only 2 studies have evaluated the molecular profile of buried glands, and only one of these was undertaken in the context of ablation. Hornick et al (2005) [22] evaluated 44 patients with Barrett's esophagus and buried glands treated by PPIs. Immunostaining for Ki67, cyclin D1 and p53 compared with adjacent areas of Barrett's esophagus. Buried glands had a significantly reduced Ki67 proliferation rate (29% vs. 49%, $P < 0.001$). There was also a trend towards reduced cyclin D1 (16% vs. 29%) and p53 (4% vs. 17%) protein expression in the buried glands. It was noted, however, that reduced proliferation rates were observed in buried glands with no opening to the luminal surface. It is unclear whether this means that decreased exposure to luminal contents in buried glands has an impact on proliferation.

After PDT ablation

In the only study looking at molecular biomarkers in buried glands following ablation, Hornick et al (2008) [90] evaluated tissue from 12 patients who had undergone PDT ablation for either HGD or intramucosal adenocarcinoma, and subsequently demonstrated buried glands. Biomarkers assessed included Ki67 proliferation, p53, cyclin D1 and DNA ploidy status. The Ki67 proliferation rate was less in the buried glands, compared to Barrett's esophagus exposed to the esophageal lumen, both before (43%) and after ablation (44%). None of the buried glands demonstrated aneuploidy compared with mild levels of aneuploidy in 73% of pre-ablation Barrett's esophagus specimens. Other biomarkers showed no difference between buried glands and pre and post ablation Barrett's esophagus. The authors concluded that buried glands following PDT may have less neoplastic potential than pre-ablation Barrett's esophagus. However, in a recent review, Gray et al (2001) pointed out that available reports have not provided sufficient information about biopsy protocols to allow assessment of their adequacy and, therefore, the frequency and importance of buried glands after endoscopic ablation remains unclear [105].

Biomarkers to predict response to ablative therapy

There have been 2 attempts to investigate whether molecular biomarkers can predict the response to ablation. Krishnadath et al (2001) [106] published in abstract only a study that looked at the ability of p53 to predict response to PDT. Ten patients (8 with HGD, and 2 with LGD) were classified as "responders" in that they had complete reversal of their Barrett's esophagus after one treatment. Nine patients (4 with HGD and 5 with LGD) who were defined as "poor responders" had persistent Barrett's esophagus. Ki67, p53, p16 and DNA-ploidy status were assessed. In specimens collected

before ablation, the poor responders had a significantly higher rate of p53 mutations compared with the responders (5/9 vs. 0/10, $P < 0.01$). p53 mutations were present in the neosquamous epithelium in 2 patients. There was no significant difference between poor responders vs. responders for p16 methylation, rate of proliferation, or ploidy status, although there was a trend towards more frequent p16 methylation in poor responders. The authors concluded that p53 mutation may play a role in predicting response to PDT. However, this study did not properly characterize the preexisting Barrett's esophagus, and a full manuscript is yet to be published.

Prasad et al (2008) [107] prospectively evaluated 126 patients with Barrett's esophagus with either HGD or intramucosal cancer. Seventy one underwent PDT ablation. The remaining 55 remained in surveillance acted as controls. Fifty (70%) responded to treatment, with no evidence of dysplasia or intramucosal cancer 3 months after PDT. Biomarkers assessed by FISH included p16 and p53. On multivariate analysis p16 allelic loss was a significant predictor of clinical response to ablation (OR 0.32; 95% CI, 0.10-0.96).

Markers that may help in the detection of residual or recurrent Barrett's esophagus.

MicroRNAs

MicroRNAs are short noncoding segments of RNA that regulate an increasing number of cellular functions [108]. A single microRNA can regulate hundreds of genes. Alterations in microRNA expression are associated with the development of certain cancers including breast, lung and gastric carcinoma [109]. MicroRNA-205 is commonly seen in esophageal squamous epithelium whilst microRNA-143 is found in columnar epithelium such as Barrett's esophagus [110]. This tissue specific expression of microRNAs means that they can be used as biomarkers to distinguish between the two types of epithelium.

There is only one published study that has looked at the microRNA expression in the setting of Barrett's esophagus and ablation [111]. In this study 9 patients with non-dysplastic Barrett's esophagus had biopsies from their Barrett's esophagus mucosa and proximal squamous epithelium before ablation with APC. MiRNA biomarkers were then compared with miRNA biomarkers from biopsies from neosquamous epithelium and normal squamous epithelium after ablation. Esophageal mucosa from 10 individuals who did not have gastro-esophageal reflux were controls. MiR-205 expression was lower in Barrett's esophagus mucosa compared to all types of squamous epithelium, and miR-143 expression was higher in Barrett's esophagus mucosa compared to all types of squamous epithelium. However, miR-143 expression was elevated in neosquamous epithelium, and

in normal squamous epithelium proximal to Barrett's esophagus epithelium, relative to expression in normal squamous epithelium in the control individuals who did not have Barrett's esophagus . This raised the possibility that the squamous mucosa in some patients with Barrett's esophagus may not be "normal" before the development of Barrett's esophagus, although more work is required to confirm this hypothesis.

Cytokeratins

Cytokeratins are proteins found within the cytoskeleton of epithelial cells. Different cytokeratins are expressed by different types of epithelium. CK-14 is expressed in the basal cells of stratified squamous epithelium and CK-8 is expressed in simple columnar epithelium [112,113]. Hence, the expression of these cytokeratins can be used as markers of different types of esophageal epithelia. The study from Dijckmeester et al (2009) that investigated miRNAs, also compared the expression of CK-8 and CK-14 between Barrett's esophagus and neosquamous epithelium [111], and found that CK-8 expression was significantly higher in Barrett's esophagus compared to neosquamous epithelium and normal squamous epithelium before and after APC ablation. Also, CK-14 expression was significantly lower in Barrett's esophagus compared to all types of squamous mucosa, and CK-8 and CK-14 expression were similar between neo-squamous epithelium and normal squamous epithelium in individuals with Barrett's esophagus. These findings were consistent with normalization of cytokeratin expression levels following conversion of metaplastic columnar epithelium to squamous epithelium.

CDX2

CDX2 is a transcription factor that plays a key role in the development and maintenance of intestinal epithelium**.** Normally it is expressed in the small bowel and large bowel mucosa, but not in the esophageal or gastric mucosa [114]. This makes it a useful biomarker for intestinal type differentiation. CDX2 is a highly sensitive marker for Barrett's esophagus even in the absence of goblet cells [115,116]. These features make it another potentially useful biomarker to distinguish between Barrett's esophagus and ablated neo-squamous tissue. However, there are no studies yet published investigating differences in CDX2 expression between Barrett's esophagus and neosquamous epithelium or neosquamous epithelium and normal squamous epithelium.

Expert Commentary

Knowing whether cancer development in Barrett's esophagus is less likely after ablation is important, as in the absence of evidence that cancer risk is reduced to a low level, ablation therapy might be regarded by health care decision makers as neither clinically effective nor cost effective. The current biomarker evidence suggests that after ablation any residual or recurrent Barrett's esophagus continues to carry a risk of neoplastic progression, although the lack of published evidence investigating the molecular profile of buried glands under neosquamous epithelium post ablation makes it difficult to draw any conclusions about the cancer risk associated with this particular mucosal subtype. Given that all ablation modalities seem to be associated with some risk of both residual and recurrent Barrett's esophagus, and long term clinical outcomes remain unknown, it cannot be assumed that cancer risk has been eliminated or reduced to a low enough level to allow patients to be treated and then discharged from clinical care. Hence, ongoing endoscopic surveillance continues to be required in all patients following ablation of dysplastic Barrett's esophagus.

Furthermore, the current evidence suggests that molecular biomarkers might only be normalized in neosquamous epithelia, so it is possible that only therapies that ablate all Barrett's esophagus will be clinically effective, highlighting the importance of either achieving complete ablation or of being able to identify patients in whom complete ablation might be achievable. There is currently only limited evidence, however, about the extent to which molecular biomarkers influence and predict an individual patient's response to ablative therapies, and with existing markers such as p16-allelic-loss we would not be able to identify patients who might have a complete response with high sensitivity and specificity.

Clinically, biomarkers have the potential to identify individuals who have undergone ablation and remain at high risk of progression to cancer, and these patients could be offered a range of options. Patients with a complete response, but with residual abnormal biomarkers within their neosquamous mucosa, could have their surveillance interval shortened to 6 months, as is currently done in some centers for patients with LGD. Patients with residual or recurrent Barrett's esophagus with abnormal biomarkers could have endoscopic resection and/or further ablation, and then regular or intensified surveillance.

However, the biggest challenge facing ablation therapies and biomarkers is that in order to detect lesions early so that they can be treated endoscopically, patients need to undergo regular surveillance, and evidence to date suggests that surveillance may not be cost effective in some of

the jurisdictions where it has been assessed [117]. Potentially, this might be overcome by offering intensified surveillance, or ablation, to patients with positive biomarkers but no dysplasia on histopathology, with discontinuation of endoscopy surveillance or lengthened surveillance intervals for biomarker negative patients. However, this approach will be constrained by the cost of biomarker testing, and needs to be assessed in cost-effectiveness models which include current treatment and outcome data from well studied surveillance programs.

5 YEAR VIEW

Molecular biomarker evidence suggests that complete ablation of both dysplasia and intestinal metaplasia is required to reduce the risk of esophageal adenocarcinoma. However, with all of the currently available ablation techniques, it is technically difficult to achieve complete eradication of all areas of intestinal metaplasia, and most studies report approximately 25-50% of patients have residual Barrett's esophagus or develop recurrence. In the future biomarkers might be useful for identifying patients that are still at high risk of progression to cancer after ablation, and these patients might be considered for intensified surveillance, or preferentially considered for endoscopic mucosal resection and/or further ablation. Ongoing research is needed to determine whether molecular biomarkers can be used to improve selection of patients for surveillance of Barrett's esophagus, as well as the timing of surveillance intervals. Cost-effectiveness studies are also required to evaluate the impact of clinical practice changes.

KEY ISSUES

- Several molecular biomarkers are associated with an increased risk of progression from Barrett's esophagus to adenocarcinoma.
- Molecular biomarker studies suggest that residual or recurrent areas of intestinal metaplasia after ablation of Barrett's esophagus are probably at risk of progression to cancer, and that only complete ablation is capable of normalizing the levels of these biomarkers.
- There is insufficient evidence from molecular biomarker studies to ascertain whether buried areas of intestinal metaplasia under post-ablation neosquamous epithelium has a reduced risk of disease progression.
- Molecular biomarkers do not currently have sufficient sensitivity and specificity to determine which patients will not respond well to endoscopic ablation.
- Whilst endoscopic surveillance of Barrett's esophagus may not be cost-effective in some of the jurisdictions where this has been assessed, using ablation and then biomarker assessment of risk to modify surveillance practice has the potential to improve the cost-effectiveness of surveillance.

TABLES

Table 1 Summary of Studies that have reported the Prevalence of recurrent or residual Barrett's esophagus following ablation

Study	Year	Number of Patients ablated	Ablative Modality	Number of Cases Complete Remission of BE (%)	Average Follow Up (months)
[43]	2011	106	RFA	51 (75% by Kaplan Meier)	36
[42]	2010	50	RFA	46 (92)	60
[36]	2010	137	RFA	106 (77)	20
[37]	2008	142	RFA	77 (54)	12
[31]	2001	58	APC	45 (78)	6
[24]	1998	17	APC	9 (53)	12
[32]	2005	19 16	APC MPEC	12 (63) 12 (75)	24
[39]	2004	12 11 10	APC Single dose PDT Fractionated dose PDT	8 (67) 9 (82) 9 (90)	12
[40]	2004	34 34	APC PDT	33 (97) 17 (50)	12
[41]	2007	138	PDT	Not reported	60
[35]	2004	15	Nd:YAG laser	6 (40)	28
[34]	1999	18	Nd:YAG laser	11 (61)	14

Abbreviations: BE, Barrett's esophagus; RFA, radiofrequency ablation; APC, argon plasma coagulation; MPEC, multipolar electrocoagulation; Nd:YAG, neodymium-doped yttrium aluminum garnet; PDT, photodynamic therapy

Table 2 Studies that reported the incidence of buried glands following ablation of Barrett's esophagus.

Study	Year	Number of Patients	Ablative Modality	Number of Cases with Buried Glands (%)	Average Follow Up (months)
[46]	2009	Single case report	RFA	Single case	9
[45]	2008	10	RFA	1 (10)	12
[27]	2007	20	APC	3 (15)	68
[39]	2004	14 12	APC Fractionated PDT	5 (36) 1 (8)	12
[24]	1999	25	APC	6 (25)	12
[44]	1999	27	APC	8 (30)	9
[29]	1996	5	PDT	2 (40)	26-44

Abbreviations: RFA, radiofrequency ablation; APC, argon plasma coagulation; PDT, photodynamic therapy

Table 3 Larger studies that have investigated the effectiveness of molecular biomarkers in assessing the relative risk of progression of Barrett's esophagus to cancer.

Study	Year	Number of Patients	Molecular Biomarkers	Mean Follow Up	Relative Risk (95% CI)
[61]	2001	256	P53 LOH	34 months	16 (6.2-39)
[65]	2000	322	aneuploidy tetraploidy	3.9 years	5 (2.7-9.4) 7.5 (4.0-14)
[67]	2004	267	aneuploidy or tetraploidy (size of clone)	2.9 years	3.9 (1.4-10.5) (for a 5 cm clone)
[67]	2004	146	P16 lesion (LOH, mutation, methylation)	4.9 years	1.3 (0.44-3.9) (for a 5 cm clone)
[66]	2001	307	Proliferation (flow cytometry to measure S phase)	56 months	1.5 (0.79-3.0) (corrected for HGD)
[75]	2008	276	Proliferation (Ki67, flow cytometry)	6.3 years	1.02 (0.99-1.05) (Hazard ratio)
[84]	2009	195	Gene-specific promoter methylation	Not stated	Depends on cut-off

Abbreviations: LOH, loss of heterozygosity; HGD, high grade dysplasia

Table 4. Studies that have investigated the presence of cancer development biomarkers in various tissues after ablation of Barrett's esophagus.

Study	Year	Number of Patients	Ablative Modality	Type of Epithelium	Molecular Biomarkers Measured	Outcome
Case control						
[98]	2008	22	PDT	NeoSE	P53 over-expression	Significantly reduced p53 expression in NeoSE ($p < 0.0$
[22]	2005	44	PPIs	Buried glands	Cell proliferation (Ki67) Cyclin D1 and p53 over-expression	Buried glands showed significantly lower proliferation r
[94]	1999	25	MPEC	NeoSE and squamous islands	Cell proliferation (Ki67), P53 over-expression, ODC activity	Elevated cell proliferation and p53 over-expression in s
Longitudinal cohort						
[99]	2009	22	RFA	NeoSE	Cell proliferation (Ki67), P53 over-expression, P16 and p53 mutation	Genetically normal
[96]	2006	20	PPIs	NeoSE	P16 mutation, P53 mutation	95% patients (19/20) NeoSE without mutations
[89]	2006	21	PDT and APC	Recurrent or residual BE	LOH analysis on APC, p16, p53, DCC, SMAD4	Persistent genetic abnormalities in recurrent or residual
[95]	2005	37	APC	NeoSE	P53 over-expression	Persistent p53 over-expression in NeoSE if present prea
[88]	2005	29	APC and PDT	Recurrent or residual BE	Cell proliferation (Ki67), P53 over-expression, DNA ploidy status	Significant reduction in DNA content abnormalities ($p =$ over-expression ($p = 0.002$) but not cell proliferation.
[87]	2000	3	PDT	Recurrent or residual BE	Cell proliferation (Ki67), DNA ploidy status, P53 mutation, P53 over-expression, P16 promoter hypermethylation	All 3 cases had 1 or more genetic markers remain positi
Selected cases						
[90]	2008	12	PDT	Buried glands Recurrent or residual BE	Cell proliferation (Ki67), DNA ploidy status, P53, cyclin D1, bcl-2, TGF-α, EGFR, AMACR over-expression	Buried glands decreased proliferation and normal ploidy Residual or recurrent BE persistently elevated proliferat significantly reduced ploidy abnormalities ($p < 0.05$)
[91]	2006	21	MPEC and APC	Recurrent or residual BE	Cell proliferation (Ki67), COX-2 over-expression, P53 over-expression	Increased Ki67 staining, COX-2 and p53 expression in r residual BE

Abbreviations: RFA, radiofrequency ablation; NeoSE, neosquamous epithelium; PDT, photodynamic therapy; BE, Barrett's esophagus; Bcl, B-cell lymphoma; TGF, transforming growth factor; EGFR, epidermal growth factor receptor; AMACR, alpha-methylacyl-CoA racemase; MPEC, multipolar electrocoagulation; APC, argon plasma coagulation; COX, cyclo-oxygenase; PPI, proton pump inhibitors; LOH, loss of heterozygosity; APC, adenomatous polyposis coli; DCC, deleted in colorectal cancer; SMAD4, mothers against decapentaplegic; ODC, ornithine decarboxylase

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