Endoscopic eradication of Barrett’s oesophagus with early neoplasia
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Properties of the neo-squamous epithelium after radiofrequency ablation of Barrett epithelium containing neoplasia

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ABSTRACT

— Objectives:
Endoscopic radiofrequency ablation (RFA) eradicates intestinal metaplasia and intraepithelial neoplasia associated with Barrett oesophagus (BO), restoring an endoscopically normal neosquamous epithelium (NSE). We evaluated the post-RFA NSE for genetic abnormalities and buried glandular mucosa (BGM).

— Methods:
Eligible patients underwent RFA for BO containing early cancer and/or high-grade intraepithelial neoplasia (HGIN) with subsequent complete histological reversion to normal NSE. At baseline, the BO was sampled by brush cytology and biopsies. At least 2 months after RFA, the NSE was sampled by brush cytology, keyhole biopsies and endoscopic resection (ER). The untreated squamous epithelium was biopsied as control. The baseline BO and post-RFA NSE were evaluated for immunohistochemical expression of Ki-67 and p53; and genetic abnormalities (DNA-FISH: chromosome 1 and 9, p16 and p53). In addition, biopsy depth was compared for biopsies from the NSE and untreated squamous epithelium. Presence of BGM in NSE was assessed with primary and keyhole biopsy, and ER.

— Results:
All pretreatment specimens from all 22 patients, showed abnormalities on immunohistochemical staining and FISH, while all post-RFA NSE specimens were normal. Post-RFA biopsies from the NSE all contained full epithelium whereas 37% contained lamina propria, a finding no different from biopsies from untreated squamous epithelium (36% lamina propria). Deeper keyhole biopsies contained lamina propria in 51%. All ER-specimens contained submucosa. No biopsy or ER-specimen contained BGM.

— Conclusions:
Rigorous evaluation of the post-RFA NSE in patients who, at baseline, had BO containing early cancer/HGIN, demonstrated neither persistent genetic abnormalities nor BGM.

INTRODUCTION
Barrett oesophagus (BO) is a metaplastic and sometimes neoplastic alteration in the oesophageal epithelium associated with injury from gastro-oesophageal reflux. Malignant degeneration in BO, when it occurs, progresses through a series of phenotypic cellular changes detected and graded on microscopy; beginning with non-dysplastic intestinal metaplasia (IM), then low- (LGIN) and high-grade intraepithelial neoplasia (HGIN), and then invasive cancer. These phenotypic changes are preceded and precipitated by an accumulation of genetic mutations and other genetic insults, resulting in cellular proliferation, cellular autonomy, and ultimately neoplasia. Selected patients with BO may be treated with an endoscopic ablation technique intended to eradicate the abnormal cells and allow restoration of a histologically normal neosquamous epithelium (NSE). While there is some data from trials of photodynamic therapy (PDT) and radiofrequency ablation (RFA) to suggest that patients converted from neoplastic BO to NSE will have a reduction in cancer progression, we know less about the genetic status of the post-ablation NSE and whether it harbors occult buried glandular mucosa (BGM) that has the potential to develop further neoplasia. For example, incidental cases of cancer arising underneath NSE after PDT and argon plasma coagulation (APC) have been reported. The most recently developed endoscopic ablation technique is RFA, with a number of reports showing favorable safety and effectiveness when applied for LGIN, HGIN, and in combination with endoscopic resection (ER) for early cancer. Thus far, all reports have shown no BGM in a large number of biopsies (i.e. total number >6,000) obtained from the post-RFA NSE. RFA may, however, result in scarring of the NSE resulting in a reduced ability to sample the histological layers beneath the epithelium [such as the lamina propria, LP], leading to false negatives for BGM. Furthermore, it is conceivable that the post-RFA NSE may harbor the same genetic abnormalities as the baseline neoplastic BO and therefore be of no lower risk for malignant transformation. Our study population underwent RFA for BO containing HGIN and/or early cancer, and subsequently achieved complete restoration of a histologically normal NSE. We compared the genetic abnormalities of the baseline BO and post-RFA NSE. We also assessed the NSE and untreated squamous epithelium with various biopsy techniques to determine depth of each biopsy and presence of BGM.

METHODS

Design
This report is derived from 3 sequential prospective clinical trials conducted at the Academic Medical Centre (AMC), Amsterdam, the Netherlands. Each study was approved by the Ethics Committee of the AMC and all patients signed an informed consent form. The first 2 trials were single-centre pilot studies (AMC-I, AMC-II), while the third was the first European multi-centre study of RFA for BO (EURO-I). The trials assessed the safety and effectiveness of RFA for treating BO containing HGIN and/or early cancer (defined as cancer limited to the upper 1/3 rd of the submucosa, aka sm1) after ER of visible lesions. Despite minor differences in methods the clinical trial protocols were generally comparable (table 1).
Evaluation of genetic abnormalities

Collection of biopsies and brush-cytology specimens

Prior to RFA (after ER where applicable) four-quadrant biopsies [every 1 cm] and brush cytology specimens were obtained from the baseline BO. Then, after RFA at least 2 months after a complete response was confirmed, four-quadrant biopsies and brush-cytology specimens were obtained from the NSE.

Evaluation of cell proliferation and p53 accumulation

Biopsies were processed routinely and stained with hematoxylin and eosin (H&E), then reviewed to classify neoplasia according to the revised Vienna classification. To evaluate proliferative activity, Ki-67 staining was applied using a mouse monoclonal antibody (Dako, Glostrup, Denmark). To assess expression of p53 protein, the p53 ab-8 mouse monoclonal antibody (Neomarkers™, Stratech Scientific Ltd, Cambridgeshire, UK) was used. Immunohistochemical staining procedures are described in detail elsewhere. For Ki-67, positive staining of the luminal surface of the epithelium was considered abnormal (scored as positive). For p53, presence of intense nuclear staining in any part of the epithelium was considered abnormal (scored as positive). Ki-67 staining and weak p53 staining of nuclei in the basal layer of (neo-) squamous epithelium was considered normal.

Evaluation of genetic abnormalities by FISH

Brush-cytology specimens were immediately processed to cytospin slides and FISH was applied with directly labeled fluorescent chromosomal centromeric probes (CEP) for chromosome 1 and 9, and the locus specific probes (LSI) for regions of 2p21 (p16) and 17p13.1 (p53) (Wysis, Downers Grove, IL). Dual color probes were used combining CEP 9 (SpectrumGreen) and LSI p16 (p21) (SpectrumOrange) as well as single probes for CEP1 (SpectrumOrange) and LSI p53 (17p13.1) (SpectrumOrange). For each FISH cytology slide, at least 100 interphase nuclei were evaluated under a fluorescent microscope (Olympus BX60, Hamburg, Germany). Damaged cells and cells with indistinct and blurry signals were ignored. To establish frequencies of artifacts resulting from background hybridization variation, probes were applied to normal squamous epithelium from 20 BO patients without neoplasia. From these counts cut-off values were calculated. A sample was considered abnormal when the number of cells with abnormal counts was equal or greater to the cut-off value.

Evaluation of post-RFA NSE tissue for biopsy depth and buried glandular mucosa

Collection of tissue specimens from NSE

After detailed endoscopic inspection of the oesophagus with high-resolution endoscopy and NBI, to ensure absence of residual columnar epithelium, patients were randomized to standard biopsies (FB-220U, Olympus, Tokyo, Japan) or to jumbo biopsies (FB-222U, Olympus, Tokyo, Japan). For jumbo biopsies, the diagnostic endoscope was switched for a therapeutic endoscope, to allow introduction of the jumbo biopsy forceps through the working channel. Four-quadrant “primary” biopsies were obtained from every 2 cm of the NSE over the entire length of baseline BO. Immediately after each primary NSE biopsy, a “keyhole” biopsy was taken from the same biopsy site. All primary and keyhole biopsies were collected in separate formalin containers for each level (i.e. 2 containers per level, 2 containers per level, 2 containers per level).
one with 4 primary NSE biopsies, one with corresponding keyhole biopsies). In addition, one set of four-quadrant "primary" biopsies was obtained from the untreated squamous epithelium of the proximal oesophagus. A NSE site that was separate from any baseline/escape ER was then selected, based on documented still images of preceding endoscopies, and marked with APC for subsequent ER. A multiband mucosectomy kit [Duette®, Cook, Limerick, Ireland] was assembled and the ER target area resected (Fig. 1). All primary and keyhole biopsies were routinely processed and stained with H&E. ER-specimens were sectioned in 2 mm slices, embedded in paraffin and a minimum of 4 serial cuts per slice were mounted on glass slides for standard H&E staining.

Figure 1. Collection of biopsies, keyhole biopsies and ER-specimen from NSE after RFA.
A: Antegrade view on an oesophagus covered with normal appearing NSE, after successful RFA of a C6M7 BD. B: Corresponding NBI image. C: Four-quadrant biopsies and keyhole biopsies are obtained every 1-2 cm of NSE. D: Endoscopic view on a biopsy site that was identified as an initial area of Barrett mucosa and that was marked with APC. E: The target area was removed using MBM. F: The ER resulted in a wound into the deep submucosal layer.

Evaluation of depth of biopsy and presence of buried glandular mucosa
Three international expert GI-pathologists from the Netherlands (FtK), Germany (MV) and the United States (RO) independently evaluated all biopsy and ER specimens in a blinded manner. They assessed the depth of each biopsy and ER specimen as either full epithelium including basal layer, lamina propria, muscularis mucosae and submucosa in each biopsy fragment. Further, each specimen was assessed for BGM.

Study outcome variables
Primary outcome variables:
• Immunohistochemical expression of Ki-67 and p53, baseline BO vs. post-RFA NSE;
• Genetic abnormalities on FISH, baseline BO vs. post-RFA NSE;
• Percentage of biopsies from post-RFA NSE containing lamina propria depth or deeper;
• BGM in primary and keyhole biopsies, and ER specimens from post-RFA NSE.

Secondary outcome variables:
– Comparison of sampling depth:
• Primary biopsies obtained from post-RFA NSE vs. untreated squamous epithelium;
• Primary biopsies vs. keyhole biopsies vs. ER from post-RFA NSE;
• Standard vs. jumbo biopsy forceps from post-RFA NSE.

Statistical analysis
Statistical analysis was performed with SPSS 16.0.2 Software for Windows. For descriptive statistics mean (±SD) was used in case of a normal distribution of variables, and median (IQR) for variables with a skewed distribution. Where appropriate Student’s t test and Mann-Whitney test were used. Pre- and post-treatment FISH score distributions were tested by x²-testing against cut-off values of controls.

RESULTS
Evaluation of genetic abnormalities
Of the 22 patients consented for genetic evaluation, 16 had undergone ER of visible lesions prior to RFA (table 1). The worst pathological grade in the residual BO before RFA was LGIN (n=3) and HGIN (n=19). After treatment, complete eradication of endoscopic BO and histological clearance of neoplasia and columnar epithelium was achieved in all patients (all had histologically normal NSE). At baseline, 90% of patients showed abnormal immunohistochemical expression for Ki-67 and 100% showed abnormal p53 expression in the biopsies of their BO-neoplasia (Fig. 2). By comparison, post-RFA NSE for all patients showed a normal distribution of lightly stained nuclei at the basal layer of the squamous epithelium, but not in the superficial layers representing a normal staining pattern (Fig. 2). At baseline, all patients (n=22) showed an abnormal probe distribution of at least one of the FISH probes tested (Fig. 3). Numerical chromosomal abnormalities were found in 60% of patients: gain of chromosome 1 (40%), or gain of chromosome 1 and 9 (20%). Loss of tumour suppressor genes was found in 90 % of patients: loss of p16 (20 %), loss of p53 (40%), and loss of p16 and p53 (30%). By comparison, the post-RFA NSE FISH counts of the centromeric probes for chromosomes 1 and 9, and the locus specific probes for p16 and p53 showed normal diploid distributions in all patients (n=22) (Fig. 3).
Figure 2. Histological images of immunohistochemical staining for p53 and Ki-67 before and after RFA.
A: (10x1.25) Intense nuclear p53 immunohistochemical staining in a group of glands of the pre-treatment biopsy.
B: (10x1.25) Ki-67 immunohistochemical staining of the pre-treatment biopsy showing extensive positive nuclear staining.
C: (4x1.25) P53 immunohistochemical staining of the post-treatment biopsy with normal staining in nuclei of the basal cells.
D: (4x1.25) Ki-67 immunohistochemical staining of the post-treatment biopsy with normal staining of nuclei of the basal cells.

Evaluation of depth of biopsy and presence of buried glandular mucosa

Of the 23 patients approached for the biopsy depth and BGM evaluation, 7 were excluded due to: baseline circumferential BO <2 cm (n=4), unrelated co-morbidity (n=2), unrelated death (n=1). The 16 eligible and consented patients had a median baseline endoscopic BO length of 7 (IQR 5-8) cm, with HGIN (n=14) or early cancer (n=2) as the worst baseline diagnosis. All patients had achieved durable eradication of neoplasia and IM after a median 26 (IQR 21-28) months follow-up. Two patients refused ER, but agreed to biopsy evaluation.

For the primary study outcome variables, a depth of lamina propria or deeper was obtained in 37% of primary biopsy specimens obtained from the post-RFA NSE. There was no BGM detected in any of the primary or keyhole biopsies, or ER specimens, obtained from the post-RFA NSE (table 2). For the secondary outcome variables, there was no difference in sampling depth when comparing primary biopsies obtained from the post-RFA NSE (37% lamina proprial vs. those obtained from untreated squamous epithelium 36% lamina propria). By comparison, keyhole biopsies from the post-RFA NSE, sampled more deeply than primary biopsies. ER specimens were deepest of all, containing submucosa in every sample. Furthermore, there was no difference between samples obtained from post-RFA NSE using standard (36% lamina propria) vs. jumbo biopsy (38% lamina propria) forceps.

Table 2. Overview of biopsy depth and presence of buried glandular mucosa.
ER: endoscopic resection specimens. NSE: neosquamous epithelium. USE: untreated squamous epithelium. Presented percentages are the mean (range) of the independent evaluations by three international, expert pathologists.
DISCUSSION

Data from clinical studies suggest that endoscopic RFA is a safe and effective modality for complete eradication of BO and associated neoplasia.\textsuperscript{15-21,23} Given that the endoscopic and histological appearance of the NSE after RFA is normal, we hypothesized that the genetic abnormalities commonly associated with neoplastic BO might be absent in the post-RFA NSE as well. Further, we sought to rigorously evaluate the post-RFA NSE with primary and keyhole biopsies as well as ER to categorically determine if occult BGM was present. We used immunohistochemical methods to compare proliferation activity (Ki-67) and accumulation of p53 protein in biopsies from the BO tissue before and after treatment. At baseline, 90% of patients had abnormal Ki-67 expression and all had abnormal p53 expression, whereas none showed any immunohistochemical abnormalities in biopsies obtained from the post-RFA NSE. We acknowledge the limitation of immunohistochemical evaluation of p53, since the results are method-dependent and do not correlate perfectly with presence of mutations. However, since increased proliferative activity and expression of p53 are known to be associated with neoplastic progression in BO, normal Ki-67 and p53 expression after RFA suggests that the NSE is more quiescent and has the potential for a reduction in risk for malignant transformation.\textsuperscript{6,7,24} We also compared numerical chromosomal changes and specific genetic abnormalities both before and after RFA by using FISH analysis of brush-cytology specimens. All patients at baseline showed an abnormal distribution of at least one of the FISH probes, whereas all samples from the post-RFA NSE showed a normal diploid signal count. One limitation of FISH is its restriction to the most frequently encountered alterations in neoplastic BO tissue, namely numerical chromosomal changes, p16 status and p53 status. We acknowledge that we did not evaluate the specific mutational status of p53/p16, the presence/absence of other genetic markers, hypermethylation, loss of heterozygosity, or alterations in the expression of other proteins. By comparison, Finkelstein et al. evaluated 21 patients with BO containing LGIN before and after RFA. Microdissection specimens from multiple targets for each patient were assessed (baseline and up to 2.5 years after RFA) for a panel of 16 allelic imbalance mutational markers affecting 1p, 3p, 5q, 9p, 10q, 17p, 17q, 21q, and 22q using quantitative fluorescent PCR with capillary electrophoresis. At baseline, all patients had multiple mutational abnormalities. RFA achieved complete eradication of all BO tissue in 15/16 patients, and all of these 15 patients demonstrated absence of the previously detected mutations.\textsuperscript{25} The finding that the post-RFA NSE has normal Ki-67 expression and negative p53 expression, and absence of pre-existing genetic abnormalities, may suggest that the NSE after RFA regenerates from a different progenitor cell than that associated with the baseline BO. Different hypotheses regarding regeneration of NSE have developed recently, including migration of adjacent squamous stem cells, repopulation from submucosal duct pluripotent cells, and deposition of circulating stem cells.\textsuperscript{26-28} Further insight of this process of NSE repopulation after RFA would be valuable to understand if eradication of BO by RFA results in clearance of genetic abnormalities of the epithelium and, thus, a reduction of the risk for developing cancer.\textsuperscript{29} Another important issue with regard to BO ablation is the possibility of occult BGM that is not readily detected on endoscopy and standard biopsy techniques. The clinical relevance of BGM is largely unknown, but one hypothetical risk is the possibility of malignant progression.
of BGM not detectable by endoscopic inspection.16,17 The presence of BGM after ablation of BO has been reported in up to 53% of patients treated with APC or PDT.20-23 This is in contrast with an absence of BGM reported in over 6,000 biopsies from the post-RFA NSE obtained in a number of well-designed clinical trials on RFA for metaplastic and neoplastic BO.11,24-27 While the absence of reported BGM after RFA may be a true-negative finding related to complete ablation, it is also possible that there is occult BGM present after RFA and that standard biopsy techniques are incapable of sampling the subepithelial tissue (lamina propria or deeper) to detect the BGM. We addressed these questions in our study and found that there was no difference in primary biopsy depth when comparing specimens obtained from the post-RFA NSE (37% lamina propria) vs. specimens obtained from the untreated squamous epithelium (36% lamina propria). This suggests that the post-RFA NSE is not more resistant to biopsy as compared to untreated tissue, and our ability to sample the sub-epithelium of the post-RFA NSE is not impaired. We addressed the issue of occult BGM by adding keyhole biopsies and ER to more deeply evaluate the post-RFA NSE. We found that keyhole biopsies sample more deeply (51% lamina propria, 31% muscularis mucosae, 5% submucosa) than primary biopsies, and that ER samples deepest of all (100% submucosa). In our primary, keyhole, or ER specimen did we identify BGM, using three independent blinded readings by three expert GI pathologists from the Netherlands, Germany and the United States. These findings make it highly unlikely that the reported absence of BGM after RFA is due to insufficient biopsy sampling depth. In a secondary outcome analysis, we compared the depth of biopsy between standard and jumbo biopsy forceps. Jumbo forceps are anecdotal believed to sample deeper aspects of the mucosa,28 however, their use requires a therapeutic endoscope which generally have inferior imaging quality and lack special optical features such as NBI. We randomized patients to undergo biopsy sampling with either a standard or jumbo biopsy forceps and no significant difference in acquired tissue depth was found. It is our opinion that it is advisable to perform follow-up endoscopy by detailed inspection of the oesophagus using a high-quality diagnostic endoscope with NBI in combination with standard biopsy forceps. In summary, the results of our study show that the genetic abnormalities present at baseline in patients with neoplastic BO are completely absent in the post-RFA NSE. Further, we determined that there is no difference in our ability to sample the sub-epithelium between the post-RFA NSE and the untreated squamous epithelium, so scarring due to RFA is unlikely. We did not detect BGM in any post-RFA NSE biopsy using a rigorous assessment with keyhole biopsies and ER. These preliminary data suggest that the NSE in patients treated with RFA for BO with intraepithelial neoplasia, may have a reduced risk for malignant transformation and that we are not missing occult BGM with our standard post-RFA follow-up biopsy regimen.

REFERENCES


