Extension of early esophageal squamous cell neoplasia into ducts and submucosal glands and the role of endoscopic ablation therapy

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ABSTRACT

BACKGROUND AND AIMS

Early esophageal squamous cell neoplasia (ESCN) is preferably treated with en-bloc endoscopic resection. Ablation might be an alternative for flat ESCN, but ESCN extension along the epithelial lining of ducts and submucosal glands (SMGs) might jeopardize ablation efficacy. Clinical studies suggest that local recurrence might arise from such buried ESCN niches after ablation. We studied human endoscopic resection specimens of ESCN to quantify ESCN extension into ducts/SMGs, and performed a prospective porcine study to evaluate depth of radiofrequency ablation (RFA) and CryoBalloon ablation (CBA) into ducts/SMGs.

METHODS

ESD specimens of flat-type ESCN from a Japanese (n=65) and Dutch cohort (n=14) were evaluated for presence and neoplastic involvement of ducts/SMGs. Twenty-seven pigs were treated with circumferential RFA (n=4), focal CBA (n=20), and focal RFA (n=3) with 4/60/9 treatment areas, respectively. After pre-specified survival periods (0h/8h/2d/5d/28d), treatment areas were evaluated for uniformity and depth of ablation and affected SMGs.

RESULTS

Neoplastic extension in ducts/SMGs was observed in the majority of lesions: 58% (38/65) in the Japanese and 64% (9/14) in the Dutch cohort. In the animal study, 33% (95%-CI 28-50) of SMGs were not affected after circumferential RFA, although the overlying epithelium was ablated. Focal RFA and CBA resulted in uniform ablations with effective treatment of all SMGs.

CONCLUSION

ESCN extends into ducts/SMGs in the majority of patients. In an animal model, focal RFA and CBA effectively ablated SMGs, while circumferential RFA inadequately ablated SMGs. Given this potential reason for recurrence, endoscopic resection should remain standard of care.

INTRODUCTION

Endoscopic submucosal dissection (ESD) is the established treatment for early esophageal squamous cell neoplasia (ESCN), since it enables controlled, *en bloc* resection and accurate histopathological assessment.[1] However, ESD is a technically challenging procedure requiring a high level of expertise. In addition, treatment-related esophageal strictures are common, in particular in extensive ESDs involving the entire esophageal circumference.[2,3]

Ablation techniques have a lower risk of complications and might offer an attractive alternative to ESD, especially for patients with early-stage disease. Radiofrequency ablation (RFA) is currently the most studied ablation technique for flat-type ESCN. Complete eradication is achieved in 84-97% of patients with flat, Lugol-unstained lesions containing intraepithelial neoplasia or mucosal cancer.[4–7] Recently, CryoBalloon ablation (CBA) has gained interest as an alternative endoscopic ablation technique. CBA appears to maintain the native esophageal mural tissue scaffold and thereby, potentially, allows for deeper ablation without jeopardizing safety.[8–10]

ESCN is a condition that often arises and expands in a patchy manner along the esophageal surface. In addition to horizontal expansion replacing the native esophageal epithelium, ESCN can demonstrate extensive vertical colonization of ducts and submucosal glands (SMGs). Ductal extension is common and has been reported in 21-38% of ESCN in Asian studies.[11,12] However, these studies either did not focus specifically on flat lesions or examined surgical resection specimens, leaving it unclear what the rate of duct extension is in flat lesions potentially amenable to ablation. Since RFA specifically aims to ablate the mucosa while preserving the integrity of the submucosa to reduce the risk of strictures,[13–15] dysplasia extending into ducts/SMGs might comprise a sheltered niche when left incompletely treated. Importantly, incomplete ablation of ducts/SMGs may escape clinical detection in particular if energy transfer across the lesion is heterogeneous, leaving some parts superficially denuded whilst the underlying ducts/SMGs remain viable. Such superficially denuded areas would be endoscopically indistinguishable from areas that have undergone transmural energy transfer with complete eradication of epithelial structures.

The first long-term follow-up results of RFA in patients with ESCN report recurrence in 14% of patients during 5 years of follow-up with progression to advanced disease stages in 5% of patients. A number of local recurrences were located in the submucosa, hidden from endoscopic view by non-dysplastic, Lugol-stained squamous epithelium.[16] At present the origin of these buried recurrences following initially successful RFA remains unclear. Hypothetically, submucosal ESCN extension into ducts/SMGs might account for these buried recurrences.[13–15] Here we aim to compare the efficacy of different ablation modalities with regard to the treatment of esophageal ducts/SMGs. To this end we

first quantified the prevalence of ESCN extension into ducts/SMGs in relation to clinicopathological parameters in ESD specimens from a Japanese and Dutch cohort. We then assessed the uniformity and depth of CBA and RFA in relation to ducts/SMGs in a prospective animal study evaluating in detail the evolution of treatment extent and efficacy over a 28-day time period.

MATERIALS AND METHODS

Histopathology ESD specimens

We conducted a retrospective analysis of 2 cohorts from Japan and the Netherlands. For the Japanese cohort, the National Cancer Center Hospital in Tokyo provided data of all patients undergoing ESD for flat or slightly depressed ESCN (Paris type 0-IIb or 0-IIc, lesions with partly elevated or excavated features were excluded) discussed at their multidisciplinary meeting between January 2008 and December 2012. This cohort has been published previously including a detailed description of the methods.[17] The Dutch cohort consisted of all patients who underwent ESD for flat or slightly depressed ESCN between April 2012 and July 2017 in 4 tertiary referral centers for endoscopic treatment in The Netherlands. Baseline patient, lesion, and treatment characteristics were collected from medical records and endoscopy and pathology reports.

The ESD specimens of the Japanese cohort were evaluated by 2 experienced gastrointestinal pathologists (MJ/RK) and the ESD specimens of the Dutch cohort were evaluated by 5 experienced gastrointestinal pathologists (JO/LB/MR/CS/MJ). All ESD specimens from both cohorts were evaluated for maximum invasion depth, lymphovascular invasion, differentiation grade, and presence and involvement of ducts/SMGs. Extension of neoplastic epithelium in at least 1 duct/SMG was scored as evidence of ductal extension (Figure 1). Ductal extension was not included in the invasion depth, unless stromal invasion arising from ducts/SMGs was found. For the Japanese cohort, the total number of ducts/SMGs present and involved by neoplasia were quantified.[17]

Animal study

A total of 27 female pigs (±80kg; Topigs, Van Beek SPF Swine Breeding BV, Lelystad, The Netherlands) were included and treated with the following ablation techniques: 1) circumferential RFA (4 pigs/4 treatment areas), 2) focal CBA (20 pigs/60 treatment areas), and 3) focal RFA (3 pigs/9 treatment areas). Survival periods differed per ablation technique. After termination, esophageal resection specimens were collected for histopathological assessment. A detailed description of the experimental procedures, follow-up and histopathological assessment is given in Supplementary Material 1.

Endpoints

Primary outcomes:

- Number of ducts/SMGs present and involved with neoplasia per ESD specimen (Japanese cohort)
- Lesions with ducts/SMGs present and involved with neoplasia (both cohorts)

- Uniform ablation, defined as a consistent maximum ablation depth in a single histopathological layer for the entire treatment area. In case of a heterogeneous ablation effect, the circumferential extent of unaffected tissue was reported as a percentage per histopathological layer (animal study).
- Percentage of completely affected SMGs per treatment area. All SMGs located in the treatment area were categorized in 3 groups: 1) Both the SMG and overlying epithelium completely affected; 2) SMG (partially) unaffected with affected overlying epithelium; 3) Both SMG and overlying epithelium unaffected (animal study).

Secondary outcomes:

- Association between tumor size, tumor location and disease stage versus number of ducts/SMGs present and involved (Japanese cohort).
- Ablation depth, defined as the deepest histopathological layer of the esophagus that was fully affected (only reported in case of a uniform ablation) (animal study).
- Maximum ablation depth, defined as the deepest layer of the esophagus affected, even if only focally affected (animal study).

Statistical analysis

No formal sample sizes were calculated for this study. R version 5.3.1 (R for Windows, R Foundation for Statistical Computing, Vienna, Austria) was used for all statistical analysis. For baseline descriptive statistics, count data were presented as numbers and percentages and continuous variables as means with standard deviations or medians with 25^{th} and 75^{th} percentiles (p25-p75) for normally or skewed distributed data, respectively. Differences in baseline characteristics between the Japanese and Dutch cohort were analyzed with the Mann Whitney U test for continuous variables and Fishers exact test for categorical variables. Outcome variables were reported as medians with 95%-CIs obtained with bootstrapping (percentile method) with 10.000 samples. For the Japanese cohort, statistical analysis of risk factors for the number of ducts/SMGs present and involved (tumor location, size and disease stage) was performed with Spearman rank correlation for continuous risk factors and Kruskal Wallis tests for risk factors with multiple categories. Fishers' exact test was used to compare the percentage of ducts/SMGs involved for risk factors with multiple categories. All analyses were corrected for multiple testing.

Ethics

For the Japanese cohort, the institutional review board granted exemption from approval. For the Dutch cohort, the Medical Ethics Review Committee of the University Medical Center Utrecht evaluated the study protocol and stated that the Medical Research Involving Human Subjects Act does not apply (reference number WAG/mb/17/004916).

For the animal study, the protocol was approved by the Animal Welfare Body Utrecht as part of a project examining new endoscopic techniques for the gastrointestinal tract approved by the Animal Experiment Committee. All animal procedures were conducted in accordance with the Experiments on Animals Act and applicable institutional, national and international guidelines and ethical standards.

The manuscript was written in accordance with the STROBE guidelines for observational studies and ARRIVE guidelines on reporting of in vivo experiments in animals.[18,19]

RESULTS

Neoplastic colonization of ducts/SMGs in ESD specimens

A total of 65 ESD specimens of ESCN were included in the Japanese cohort and 14 in the Dutch cohort (Table 1). For the Japanese ESD specimens, all ducts/SMGs were counted. The median (95%-CI) number of ducts/SMGs present per ESD specimen was 10 (5-21), and median 1 duct/SMG (1-2) had neoplastic involvement. The number of involved ducts/SMGs increased along with the number of ducts/SMGs present (ρ=0.76, *P*<0.001; Figure 2A). Of the 65 ESD specimens, 53 (82%) had ducts/SMGs and 38 (58%) had neoplastic extension .

We assessed the potential effect of tumor size, tumor location and disease stage on ducts/SMGs in the Japanese cohort. Regarding tumor size, ducts/SMGs were present and showed neoplastic involvement for all tumor sizes. The number of ducts/SMGs involved tended to increase with tumor size, but with significant variation between cases (ρ=0.23, *P*=.06; Figure 2C). For tumor location, we found no association with the number of ducts/SMGs present or involved (Kruskal Wallis, *P*=0.89/*P*=0.65, data not shown). Regarding disease stage, we found no neoplastic involvement of ducts/SMGs in any of the high-grade intraepithelial (HGIN) lesions, whereas all more advanced disease stages (i.e. T1m2 and beyond) revealed involved ducts/SMGs (Figure 2D). The median number of colonized ducts/SMGs increased with deeper tumor invasion (Kruskal Wallis, *P*=0.02; Figure 2E), whereas the median number of ducts/SMGs present did not differ per disease stage (Kruskal Wallis, *P*=0.23; Figure 2F).

For the Dutch cohort, presence and neoplastic involvement of ducts/SMGs were scored per ESD specimen. Ducts/SMGs were present in 79% (11/14) of the ESD specimens and neoplastic extension in 64% (9/14). Involved ducts/SMGs were found in 1/2 HGIN (50%), 4/4 mucosal (100%), and 4/5 submucosal (80%) lesions with ducts/SMGs present (Figure 2G).

Animal study

Between January 2019 and February 2020, all ablations in all 27 pigs were successfully performed and all pigs survived the aimed follow-up periods without adverse events.

Circumferential RFA

Circumferential RFA (4 pigs, 1 treatment area per animal) resulted in a heterogeneous treatment efficacy and skipped zones were observed in all treatment areas. For the epithelial layer, a maximum of 20-50% of the circumference was not effectively ablated, and 50-80% for the submucosa.

Importantly, 33% of SMGs (95%-CI 28-50) remained viable after treatment, although the overlying epithelium had completely sloughed off (labeled as shoulder region; Figure 3A/B and D/G).

Eight percent of SMGs (95%-CI 0-22%) was viable and had viable overlying epithelium (Unaffected; Figure 3A/B and E/H). The remaining 54% (95%-CI 47-62) had complete ablation of SMGs and overlying epithelium (Deep Ablation; Figure 3A/B and C/F).

Due to variable ablation depth within the treatment areas, no single efficacy score was reported per esophageal wall layer. The maximum (focal) ablation depth was the upper half of the circular layer of the deep muscle (muscularis propria) for all treatment areas.

Focal CBA

Focal CBA (20 pigs, 3 treatment areas per animal) resulted in a uniformly ablated treatment area with a sharp vertical demarcation line between treated and untreated areas (Figure 4A). No skipped zones were observed.

Focal CBA resulted in complete ablation of all SMGs in all 60 treatment areas. At 0 and 8 hours, early ablation effects were seen in SMGs, including loss of cell borders and nuclei, and subepithelial edema. At days 2 and 5, all SMGs in the 24 treated areas showed complete necrosis. At day 28, no SMGs were found in the 12 treated areas and complete fibrosis of the submucosal layer was observed (Figure 5a; Supplementary Table 1b).

Ablation of all treatment areas was homogeneous, with complete ablation of the entire epithelium and submucosa. Maximum ablation depth was seen at day 5 with focal necrosis into the adventitia (present in 8% of treatment areas). At this point, the epithelium, mucosa, submucosa, and muscularis propria all showed complete necrosis in all 12 treatment areas. After 28 days, re-epithelialization had occurred and extensive fibrosis was seen in the submucosa and muscularis propria, and focal fibrosis in the adventitia (Figure 5a; Supplementary Table 1b).

Focal RFA

Focal RFA (3 pigs, 3 treatment areas per animal) resulted in a homogeneous ablation with a sharp vertical demarcation line between the treated and untreated areas without skipped zones in all 9 treatment areas (Figure 4B).

Complete ablation of all SMGs was seen in all 9 treatment areas. At day 2, loss of cell borders and nuclei was seen in the SMGs and subepithelial edema was observed. At day 5, full necrosis of all SMGs was seen. After 28 days, the submucosa was fibrotic and there were no remaining SMGs in the 3 treated areas (Figure 5b; Supplementary Table 1b).

The ablation depth was homogeneous for all treatment areas and both the epithelium and the submucosal layer were adequately ablated. The ablation effect was most prominent 2 days after treatment. The deepest visible effect was focal necrosis in the muscularis propria. In all cases, the epithelium, mucosa and submucosa showed complete necrosis. After 28 days, re-epithelialization had

occurred and fibrosis was seen along the full depth of the submucosa (Figure 5b; Supplementary Table 1b).

DISCUSSION

Here we have investigated, through a paired clinical and animal study, the potential origin of subsquamous recurrences in patients after initially successful RFA of ESCN. We focused our analysis on patients with flat ESCN, eligible for ablative treatment. Our detailed analysis reveals near universal neoplastic colonization of ducts/SMGs. Importantly, our animal data show extensive heterogeneity within the ablated segment with regard to vertical energy transfer in c-RFA. In particular, we reveal that a substantial proportion of SMGs remains viable following c-RFA, while the overlying epithelium was adequately ablated. These data lend support to the hypothesis that buried recurrences may arise from inadequately treated neoplastic niches in esophageal ducts/SMGs. By contrast, focal ablation using RFA or CBA resulted in uniform ablations in all animals with adequate treatment of both the epithelial and submucosal layer including all SMGs.

The evaluation of ablation therapy for early ESCN is clinically relevant because ESCN is common, especially in Asia where wide-spread ESCN screening is performed, and current treatment options are limited.[20,21] ESD, the standard of care, requires a high level of expertise, is time-consuming and has a substantial risk of stenosis after circumferential resection.[1–3] Ablation therapy might overcome these drawbacks, especially for more extensive lesions. However, the main drawback of ablation therapy as compared to ESD, is that no tissue is acquired for histopathological assessment. Accurate pre-treatment assessment of true invasion depth is impossible since biopsies only sample a fraction of the lesion and may therefore underestimate disease stage. By contrast, ESD enables an en-bloc resection of the lesion, which allows for accurate histopathological assessment and may guide follow-up and additional treatment.

RFA is the most widely investigated ablation technique for ESCN with complete response reported in 84-97%.[4–7] However, long-term follow-up results for RFA in ESCN are worrisome, since a significant proportion of patients developed recurrence during follow-up (14-20%).[7,16] In contrast, a recent meta-analysis on ESD for early ESCN reported a pooled local recurrence risk of 0.3% (1/398).[22] Furthermore, some patients developed subepithelial, buried recurrences after RFA.[16] Due to this silent progression, hidden from endoscopic view, lesions were not picked up during follow-up endoscopies, yet only detected at advanced stages. Since these lesions have not been observed in treatment-naïve patients, a causal relation with RFA treatment appears plausible. Notably, this observation is specific for ESCN and has not been reported in long-term follow-up results on RFA for Barrett's esophagus.[23,24]

Based on these observations, it has been hypothesized that ESCN may extend into ducts/SMGs and efficacy of RFA may be too superficial for effective ablation of all ducts/SMGs.[7,17] Especially when neoplastic ducts/SMGs persist, while the overlying epithelium is effectively ablated and re-epithelialized with non-dysplastic squamous epithelium, this may lead to buried disease during follow-up.

In the first part of this study, we determined the relevance of neoplastic extension in ducts/SMGs by assessment of its prevalence in patients with flat ESCN. Since ESCN has a strong geographic distribution, we assessed the prevalence both in a Japanese and Dutch cohort. The results were comparable: the majority of lesions showed neoplastic extension in ducts/SMGs (58% and 64% in the Japanese and Dutch cohort, respectively). Previous studies acknowledged that neoplastic extension in ducts/SMGs is common, but were solely conducted in Asian countries.[11,12] Moreover, in line with a previous report,[11] neoplastic extension in ducts/SMGs increases with disease stage. Since ablation therapy is indicated for flat-type neoplasia, the incidence of neoplastic extension in ducts/SMGs for flat HGIN is of major relevance. Although the number of lesions with HGIN in our cohorts was limited, one of the two HGIN lesions in the Dutch cohort showed invasion of ducts/SMGs. Therefore, no specific disease stage can be considered to be completely free of neoplastic extension in ducts/SMGs.

Next, we evaluated the effect of ablation therapy on ducts/SMGs in a porcine animal model. Since the two subepithelial recurrences in the RFA follow-up study were observed after initial treatment with c-RFA,[16] effects of c-RFA were our main interest. We found that 33% (95%-CI 28-50) of SMGs within the treatment areas were unaffected while the overlying epithelium was adequately ablated (shoulder regions). The extent of superficial ablation on macroscopic images thus *over*estimates the extent of effective submucosal penetration, as large swathes of inadequately treated submucosa demonstrated superficial epithelial denudation. This is especially concerning given that these regions will not be appropriately recognized as inadequately treated during Lugol's endoscopy. This observation may explain the risk for subepithelial recurrences after c-RFA.

In contrast to c-RFA, focal ablation using RFA or CBA ablated sufficiently deep to adequately eradicate all ducts/SMGs in the animal model, and this was confirmed by absence of ducts/SMGs in the treated areas after 28 days of follow-up. Importantly, in contrast with c-RFA, ablation depth was uniform in the entire treatment area. Therefore, focal ablation therapy with either RFA or CBA may potentially be effective for small, early ESCN. However, clinical long-term follow-up studies are warranted prior to widespread clinical application.

An important strength of our study is the combination of human data to quantify the problem of neoplastic extension in ducts/SMGs and animal data to assess the uniformity and depth of ablation. The human study was based on a large Japanese cohort, where all ducts/SMGs were separately counted, combined with the first report on neoplastic extension in ducts/SMGs in a European population. In contrast with previous studies, only flat-type lesions resected with ESD were selected. For the prospective animal study, treatment procedures were standardized and performed by experienced endoscopists. If possible (i.e. after focal ablation), multiple survival periods were used to gain insight in injury and recovery patterns over time. Interim evaluations showed homogeneous results between and within animals, allowing our study design to be adjusted to minimize animal use.

Important limitations should also be addressed. Despite the fact that only flat-type lesions were selected, the first part of the study in human ESD specimens was limited by a relative small number of lesions with HGIN, which might be considered the main indication for ablation therapy. The limited number of lesions hampered subgroup analysis for risk factors of neoplastic extension in ducts/SMGs in the Dutch cohort. Likewise, the second, animal part of the study has limitations impeding translation to clinical practice. First, even though the porcine esophageal anatomy mimics human anatomy in important respects, in particular the presence of ducts/SMGs, and is a frequently used model to evaluate esophageal endoscopic interventions in the preclinical phase, important differences with the human esophageal wall structure hamper direct translation of ablation depth.[25] In pigs, SMGs are widely present in the proximal esophagus and gradually decrease towards the distal esophagus, while in humans the prevalence of SMGs varies widely in number and distribution between and within individuals and clusters of SMGs at either end of the esophagus are described.[25,26] Ideally, the animal data would be correlated with a human pre-esophagectomy study. However, the large variation in number and distribution of SMGs and the limited space for ablation therapy in pre-esophagectomy patients to not interfere with operation techniques limits the chance of finding ducts/SMGs in the ablated area. Furthermore, no ESCN was present in the animal model and the survival pattern of neoplastic cells after ablation therapy might be different. Secondly, although the absence of ducts/SMGs after 28 days would be the ultimate evidence of effective treatment, the maximum survival time for c-RFA was limited to 2 days because of expected substantial risk of severe strictures after circumferential treatment in pigs.[25,27] Thirdly, we tested only a single dose per technique and outcomes may be different for other dosages. In addition to this, current aggressive prophylactic stricture management may allow higher RFA dosages. If higher dosages of RFA lead to deeper ablation, the effects on SMGs may also be more pronounced. However, in the current study doses were selected based on most recent advices for clinical practice for each technique. For c-RFA, a single application of 1x10J was used, which is the currently advised ablation regimen for treatment of early ESCN.[5,16] Although double c-RFA regimens may lower the risk of skipped zones, (subepithelial) recurrences in clinical long-term follow-up studies were observed after single as well as double c-RFA regimens.[7,16] Lastly, circumferential CBA was not included in this study, because currently no large-area CBA device is available. First feasibility studies have been performed in patients with Barrett's esophagus, but optimal dosing remains to be determined.[28,29]

Despite these limitations, several clinically relevant conclusions can be drawn. Endoscopists should be aware that the majority of early ESCN lesions extend vertically into ducts/SMGs. In addition, the use of c-RFA for early ESCN should be discouraged because of the risk of persistent neoplasia in ducts/SMGs hidden beneath superficially affected areas (shoulder regions). To determine the role of focal RFA and CBA for early ESCN, clinical studies with long-term follow-up data are required prior to

clinical application, as (subepithelial) recurrences may present late. If circumferential CBA becomes available, histopathological studies to assess the effects of circumferential CBA on ducts/SMGs are indispensable before initiation of clinical ESCN studies.

In conclusion, extension of early, flat-type ESCN into ducts/SMGs occurs in the majority of patients. Circumferential RFA resulted in heterogeneous depth of ablation and persisting SMGs underneath adequately treated epithelium in an animal model. This may be a reason for recurrence and suggests that endoscopic resection should be preferred over circumferential RFA for treatment of early ESCN. Focal CBA and RFA on the other hand, appear to effectively ablate SMGs, yet should be further evaluated in long-term clinical follow-up studies before widespread clinical application of focal ablation for early ESCN.

Table 1. Baseline characteristics of the Japanese and Dutch cohort.

	Japanese Cohort (n=65)	Dutch Cohort (n=14)
Age, median (p25-p75)	68 (63-74)	68 (64-72)
Male sex*, n (%)	58 (89)	6 (43)
Location*, n (%)		
Cervical esophagus (15-19)	1 (2)	1 (7)
Upper thoracic esophagus (20-24)	0 (0)	2 (14)
Middle thoracic esophagus (25-29)	39 (59)	5 (36)
Lower thoracic esophagus (30-40)	25 (39)	6 (43)
Primary Paris classification*, n (%)		
0-IIb	4 (6)	11 (79)
0-IIc	60 (94)	3 (21)
Tumor size in mm, median (p25-p75)	38 (32-43)	35 (20-40)
Resection size in mm, median (p25-p75)	45 (40-50)	40 (27-48)
Disease stage*, n (%)		
HGIN/M1	7 (11)	4 (29)
M2	31 (48)	2 (14)
M3	21 (34)	3 (21)
SM1	3 (5)	3 (21)
SM2/3	2 (3)	2 (14)
Differentiation grade*, n (%)		
Good	0 (0)	0 (0)
Moderate	56 (86)	6 (42)
Poor	2 (3)	4 (29)
N/A	7 (11)	4 (29)
Lymphovascular invasion*, n (%)	6 (9)	3 (21)

* The two cohorts differed significantly for sex, tumor location, primary Paris classification, disease stage, differentiation grade and presence of lymphovascular invasion.

HGIN High-grade intraepithelial neoplasia.

Figure legends

Figure 1. Overview vertical duct extension in ESCN

Histopathological images of vertical duct extension in ESCN. (A) Low power photomicrograph showing an expanding ESCN (T1m2, arrows) colonizing a submucosal gland duct (asterisk). Arrowheads indicate adjacent non-dysplastic squamous esophageal epithelium and the § indicates a submucosal gland complex. (B) Magnification of dashed box in A showing ESCN colonizing submucosal gland duct. Asterisk indicates ESCN and arrowheads indicate non-dysplastic duct epithelium.

Figure 2. Presence and neoplastic involvement of ducts and/or submucosal glands

SMG *submucosal gland;* ***HGIN*** *high-grade intraepithelial neoplasia;* ***ESD*** *endoscopic submucosal dissection.*

Presence (in green) and neoplastic involvement (in orange) of ducts and/or submucosal glands for the Japanese (A-F) and Dutch (G) cohort. (A) The number of ducts/SMGs with neoplastic involvement increases with the number of ducts/SMGs present (ρ=0.76, *P*<0.001); (B) No statistically significant association between tumor size and the number of ducts/SMGs present was found (ρ=0.10, *P*=.42); (C) Tumor size was also not significantly associated with the number of ducts/SMGs involved (ρ=0.23, *P*=.06); (D) For HGIN, none of the ducts/SMGs present were involved, whereas all more advanced disease stages showed neoplastic involvement of ducts/SMGs; (E) The number of involved ducts/SMGs present did not differ per disease stage (Kruskal Wallis, *P*=0.23); (F) However, the number of ducts/SMGs involved was significantly lower for HGIN versus T1m2 (Kruskal Wallis, *P*=0.002) and T1m3 (Kruskal Wallis, *P*=0.003); (G) Presence and involvement of ducts/SMGs in the Dutch Cohort.

Figure 3 Histopathological assessment of submucosal glands after circumferential radiofrequency ablation

Histopathological images of submucosal glands after circumferential radiofrequency ablation (RFA). (A) Macroscopic image of the porcine esophagus after longitudinal opening revealing a heterogeneous treatment effect. The dashed area indicates the treated area. The unaffected area marks normal epithelium within the circumferential RFA treatment zone. The asterisk indicates the section used for images B-H. Bar indicates 1 centimeter. (B) Low power microscopic image of the section indicated with an asterisk in A. Circumferential RFA treatment area (C), shoulder region (D), and unaffected area (E) are shown. Dashed rectangles indicate high power areas shown in C-H. (F) Ablated submucosal gland. Asterisk shows necrotic submucosal gland acini and a polymorphic infiltrate. (G) Submucosal gland in

shoulder region shows mild polymorph infiltrate (arrowheads), but with intact epithelial gland lining. (H) Submucosal gland in unaffected area shows entirely normal submucosal gland.

Figure 4 Histopathological images of the sharp border between treated and untreated areas after focal ablative therapy

The dashed line marks the sharp border between treated and untreated areas revealing clear differences in the epithelium, submucosa and submucosal glands 2 days after focal CryoBalloon ablation (A) and focal radiofrequency ablation (B). Asterisk indicates necrotic submucosal gland.

Figure 5a Macroscopic and microscopic effects of focal CryoBalloon ablation per layer of the esophageal wall per survival period

Time course focal CryoBalloon ablation: (A-E) Macroscopic overview of opened porcine esophagus per survival period. Bar indicates 1 centimeter. (F-J) Low power microscopic overview of treatment areas. (F-G and K-L) Early changes with retained architecture of submucosal glands (SMGs), but with loss of cell boundaries in SMG cells (K-L) and subepithelial edema (G - arrowhead). (H-I and M-N) Complete necrosis of squamous epithelium (asterisk) and submucosal glands and extensive submucosal edema. (J) Reepithelization and remodeling of esophageal wall layers with extensive fibrosis of the submucosa and absence of SMGs.

Figure 5b Macroscopic and microscopic effects of focal radiofrequency ablation effects per layer of the esophageal wall per survival period

Time course focal radiofrequency ablation: (A-C) Macroscopic view of the opened porcine esophagus per survival period. Bar indicates 1 centimeter. (D-F) Microscopy of treatment areas revealing necrosis and sloughing of squamous epithelium (asterisk) and submucosal glands after 2 days (D and G) and 5 days (E and H) and reepithelization and extensive submucosal fibrosis and absence of submucosal glands after 28 days (F).

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