DOCTORAL THESIS

Potential roles of gastro-esophageal reflux in patients with superficial esophageal squamous cell carcinoma without major causative risk factors

（食道扁平上皮癌発症低リスク患者群の臨床病理学的特徴とその発癌における逆流性食道炎の影響）

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ORIGINAL ARTICLE—ALIMENTARY TRACT

Potential roles of gastroesophageal reflux in patients with superficial esophageal squamous cell carcinoma without major causative risk factors

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Abstract

Background Cigarette smoking, alcohol consumption, and Lugol-voiding lesions (LVLs) are the major causative risk factors of esophageal squamous cell carcinoma (ESCC); however, reports on ESCC cases unrelated to these risk factors are very limited. Here, we investigated the clinicopathological features and etiology of such cases.

Methods We retrospectively analyzed 704 consecutive superficial ESCC tumors of 512 patients who were treated with endoscopic submucosal dissection. The enrolled patients were divided into two groups—the very low-risk (VLR)-group and risk (R)-group—based on the presence of the abovementioned risks. Clinical, endoscopic, and pathological characteristics and genetic findings were assessed in both groups.

Results The VLR-group consisted of 21 (4.1%) patients, who were characteristically female. Patients in the VLR-group presented gastroesophageal reflux disease (GERD), hiatal hernia, and non-open-type atrophic gastritis, and were negative for *Helicobacter pylori*. We found unique endoscopic features—frequently observed in the posterior wall of the middle thoracic esophagus—with a linear shape that closely resembled the erosion-like form of GERD. Additionally, histopathological examination showed that these tumors presented atypical nuclei limited to the basal and parabasal layer, sequential to the surrounding changes that presented pathological chronic inflammation of esophagitis. Evaluation of somatic mutations in cancer-related genes using next-generation sequencing revealed that the positive carcinogenic potential (*TP53* mutation) of the tumors was relatively frequent in the VLR-group.

Conclusions Our study suggests that ESCC without major causative factors is related to GERD, with no remarkable oncogenic difference.

Keywords Endoscopic submucosal dissection · Esophageal · Squamous cell carcinoma · Next-generation sequencing · Gastroesophageal reflux · *TP53*

Abbreviations

ESD	Endoscopic submucosal dissection
ESCC	Esophageal squamous cell carcinoma
EAC	Esophageal adenocarcinoma
LVLs	Lugol-voiding lesions
GERD	Gastroesophageal reflux disease
FSSG	Frequency scale for the symptoms of GERD
SD	Standard deviation
CRT	Chemoradiation therapy
NGS	Next-generation sequencing
LMD	Laser microdissection
COSMIC	Catalogue of Somatic Mutations in Cancer

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VLR-group	Very low-risk group
R-group	Risk group

Introduction

Esophageal carcinoma is the eighth most common cancer and the sixth most common cause of cancer-related deaths worldwide [1–3]. It is about three times more common in males than in females [4, 5]. Esophageal squamous cell carcinoma (ESCC) is a predominant histological subtype of esophageal cancer in Asian countries, whereas esophageal adenocarcinoma (EAC) predominates in Western countries [6]. There is a strong and causal relation between gastroesophageal reflux and EAC. In contrast, the major causative factors for ESCC are tobacco smoking and alcohol consumption, which in combination exhibit synergistic effects [7, 8]. Further, a recent prospective cohort study of patients who had undergone endoscopic resection for ESCC demonstrated a strong association between the cumulative incidence of metachronous ESCC and the grade of esophageal Lugol-voiding lesions (LVLs), as assessed by Lugol chromoendoscopy [9, 10]. Multiple dysplastic lesions assessed as LVLs also increase the risk of multiple ESCCs, and this phenomenon has been explained by the field cancerization theory. Therefore, a careful follow-up is needed to identify ESCC in its early stage for those patients who consume alcohol, smoke, and have been diagnosed with multiple dysplastic lesions.

However, patients with ESCC without evident history of exposure to the major risk factors are sometimes encountered, and the mechanism of carcinogenesis in such cases remains unclear. In the present study, we aimed to determine the features of patients with early ESCC—treated by endoscopic submucosal dissection (ESD)—without a history of habitual smoking, drinking, and multiple LVLs.

Materials and methods

Study design and participant group classification

This was a single-center retrospective study using a prospectively maintained dataset. We collected data of 704 consecutive ESCCs in 512 patients treated with ESD at the Yokohama City University Medical Center between August 2002 and December 2018. All participants were Japanese. Smoking and drinking histories and other epidemiological data, such as history of previous cancer, surgery history, and family history of esophageal cancer, were available for all cases from the patient medical records and interviews. As the grade of LVLs is a major risk factor, in addition to alcohol consumption and smoking habits, the LVLs were evaluated in preoperative endoscopy and graded based on the number of LVLs per endoscopic view (grade A: no lesions; B: 1 – 9 lesions; and C: $\geq$ 10 lesions) [9, 10]. The patients were classified into two groups, i.e., the very low-risk group (VLR-group, n = 22), which included patients without habitual current or former exposure to smoking or alcohol consumption and with LVLs of grade A; and the risk group (R-group, n = 682), which comprised patients who were current or former habitual users of tobacco and/or alcohol, and/or had LVLs of grade B or C.

Indication of endoscopic resection

Preoperative endoscopic diagnosis for ESD was obtained by conventional endoscopy, chromoendoscopy with iodine staining, and since 2008, magnified endoscopy with narrow-band imaging (ME-NBI, Olympus Corp, Tokyo, Japan). Definite indication for ESD was the clinical depth of tumor for cT1a EP or LPM, and relative indication was cT1a MM or cT1b SM1, due to an elevated risk of lymph-node metastasis [11].

Endoscopic resection procedures

The recent development of therapeutic endoscopic procedures has enabled the en bloc and R0 resections of esophageal squamous neoplasia [12, 13]. The specimens resected by ESD provide ideal materials for accurate pathological diagnosis—regarding lateral development, tumor depth, and comparison with the endoscopic findings—and cancer-related gene analysis. Therefore, in the present study, all participants had ESD.

Patients were sedated for endoscopic procedures using intravenous midazolam and pentazocine. For the ESD, a single-channel upper gastrointestinal endoscope with a water jet system (GIF-Q260J; Olympus Medical Systems, Co, Tokyo, Japan) and a standard electrosurgical generator (ICC 200 or VIO300D; ERBE, Tübingen, Germany) were used with CO2 insufflation. A dual knife (KD-650Q, Olympus Medical Systems, Co.) and an insulation-tipped knife-nano (KD-612, Olympus Medical Systems, Co.) were used as the main electrosurgical knives. The tumor margins were delineated with iodine staining and marking dots were placed outside the margins of the tumor using the electrosurgical knife. The lesion was lifted by injection of a hyaluronic acid solution into the submucosal layer, and then submucosal dissection was performed.

Terminology and definition related to treatment

En bloc resection was defined as one-piece resection of a target area on the endoscopy, and R0 resection was defined as en bloc and tumor-free margins. Intramucosal tumors (T1a) were further distinguished as tumors invading the epithelium (T1a-EP), lamina propria (T1a-LPM), or muscularis mucosae (T1a-MM). Submucosal tumors (T1b) were divided into invasion of the submucosal layer to $\leq$ 200 μm (T1b-SM1) and tumors invading beyond this threshold in the submucosa (T1b-SM2-3). Curative resection was considered as a combination of criteria such as R0 resection of a superficial lesion with histology, no more advanced than pLPM, with no lymphovascular invasion, according to the Guidelines for Diagnosis and Treatment of Carcinoma of the Esophagus edited by the Japanese Esophageal Society [11]. The circumferential location of the esophagus was divided into four areas every 90 degrees, as anterior (11–2 o'clock direction), right (2–5), posterior (5–8), and left wall (8–11). The orientation was given with the left wall being the direction in which water accumulated in the patient's left lateral decubitus position. The position of the center line of the tumor was used as the orientation for determining the cross-sectional location of the tumor. Circumferential lesions were excluded from this localization analysis.

Reflux esophagitis was defined endoscopically and classified according to the Los Angeles (LA) classification (grades A, B, C, and D) with Japanese modification [14]. The hiatal hernia was evaluated endoscopically by grading the gastroesophageal flap valve (GEFV) using the Hill classification (Hill grade I–IV) [15]. The Hill classification has been proven to be reproducible and provides useful information for evaluating patients with suspected gastroesophageal reflux disease (GERD). In our study, a diagnosis of atrophic gastritis was made if atrophy was identified as open type upon using Kimura and Takemoto criteria [16]. We diagnosed GERD according to the guidelines described in the GERD treatment flowchart of the Japanese Society of Gastroenterology [17]. We also used the FSSG (frequency scale for the symptoms of GERD) questionnaire for the evaluation of symptoms of GERD [18]. The 24-h MII-pH monitoring test was not conducted for evaluation of GERD because of its high invasiveness.

Evaluations for clinicopathological and treatment outcomes

We evaluated clinical features, such as sex, age, body mass index, family history of ESCC, synchronous ESCC, metachronous ESCC, previous history of cancer, previous history of head and neck cancer, history of surgery, past history of chemoradiation therapy (CRT) for ESCC, gastroesophageal reflux (GERD), hiatal hernia, and atrophic gastritis of patients in the VLR- and R-groups. We also analyzed differences in tumor characteristics of lesions, such as macroscopic type, tumor location, tumor and specimen size, and treatment outcome of the VLR- and R-groups. The histological diagnosis was in accordance with the Japanese Classification of Esophageal Cancer (11th edition) [11]. Resected specimens were fixed in 10–20% formalin, serially sectioned at 2 mm intervals, and assessed using histological mapping. Expert pathologists were engaged to assess the macroscopic type, tumor size, depth of invasion, lymphatic and vascular involvement, and lateral and vertical margins. Immunostaining of specimens was performed using p53-specific antibody (Anti-Human p53 Protein, Clone DO-7).

Genetic analysis

We investigated the formalin-fixed paraffin-embedded (FFPE) resected specimens to confirm the differences between the somatic mutations in cancer-related genes using next-generation sequencing (NGS)-based target sequencing with the Ion Proton-based cancer panel platform. To reveal the somatic aberrations, we introduced laser microdissection (LMD) technology, which allowed us to precisely obtain small neoplastic samples. FFPE blocks were cut into 4 μm-thick sections for the detection of tumor tissue and background non-tumor tissue and into 15 μm-thick sections for laser microdissection. DNA from normal mucosa was used as a control for the detection of variants in tumors (tumor–normal paired analysis) performed after LMD. The quantity and quality of the extracted DNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher). Additionally, the quality of the pooled libraries was checked using the 4150 Tape Station instrument of the D1000 ScreenTape System (Agilent Technologies, USA). Data analysis was performed using Ion Reporter software; allele frequency (AF) > 2% and coverage (Cov.) Results > 200 were defined as positive. Mutations listed on COSMIC (Catalogue of Somatic Mutations in Cancer, http://www.sanger.ac.uk/cosmic) were classified as "putative driver mutations." We used a ready-made gene panel (Ion AmpliSeqCancer Hotspot Panel v.2, Thermo Fisher Scientific, Waltham, MA, USA) to amplify 50 cancer-related target genes. A total of 2790 hotspot mutations of the 50 cancer-related genes are reported in the COSMIC database (Supplementary Fig. 1).

We could not perform cancer-related gene target sequencing for four cases because the lesions treated before 2015 were judged not suitable for analysis. These lesions were originally fixed in 20% formaldehyde, which made it technically difficult to extract the DNA.

Follow-up and additional treatment

Esophagogastroduodenoscopy (EGD) surveillance after ESD was routinely performed in the following way. For curative resection, EGD was performed 3 months after ESD, and thereafter, semi-annual endoscopy was conducted. In cases of non-curative resection, surgery, CRT, or radiation therapy was recommended depending on the patient's general condition. Metachronous ESCC was defined as ESCC other than local recurrence detected in surveillance EGD.

Ethics statement

The study and its protocols were approved by the ethical review boards of our hospital (Yokohama City University Certified Institutional Review Board; A180524005, D1602024). All patients were informed of the risks and benefits of treatments before they underwent the procedures. Informed consent was obtained from all patients included in the study.

Statistical analysis

Statistical analysis was performed with JMP 14 (SAS Institute Inc., Cary, NC, USA). Proportions of categorical variables were compared using the two-sided Fisher's exact test or chi-squared test. Continuous variables were compared using the Wilcoxon–Mann–Whitney test. A p-value < 0.05 was considered significant. Overall survival rates were calculated using the Kaplan–Meier method with the log-rank test.

Results

Characteristics of ESCC patients without risk factors

Of a total of 512 patients with superficial ESCC, 21 were in the VLR-group, and the remaining 491 patients were in the R-group. Clinical characteristics of the patients in the VLR- and R-groups are compared in Table 1. Interestingly, all of the patients in the VLR-group were female and the proportion of females was significantly higher than in the R-group (100% vs. 11.4%, $p < 0.001$). In the VLR-group, no cases were found with family history of ESCC, synchronous ESCC, or previous history of head and neck cancer, but without statistical significance compared with the R-group. The presence of GERD (76.2% vs. 25.5%; $p < 0.001$), hiatal hernia (85.7% vs. 28.7%, $p < 0.001$), and non-open-type atrophic gastritis (81.0% vs. 34.6%, $p < 0.001$) was significantly higher in the VLR-group compared with that of the R-group.

During the median follow-up period of 42 months (range 1.1–194 months), the cumulative incidence of metachronous ESCC did not significantly differ between the groups (4.8% vs. 13.4%; $p = 0.339$), and no patient died in the VLR-group during the observation period (0% vs. 9.2%; $p = 0.242$).

Comparison of tumor characteristics between VLR- and R-groups

Table 2 shows the tumor lesion-based comparative analysis between the two groups. Macroscopic type, median tumor size, and median specimen size did not significantly differ between the groups. However, we found a significant difference in circumferential location ($p = 0.0029$), but not in vertical location of the esophagus ($p = 0.955$). Tumors of patients in the VLR-group were frequently observed in the 5–8 o'clock location; this observation will be further discussed in later subsections. We also found a significant difference in tumor depth between the two groups ($p = 0.0019$); most of the tumors in the VLR-group were non-invasive and remained in the EP with no submucosal invasion.

Clinical features of patients within the VLR-group

We additionally investigated specific features of the VLR-group based on responses of the patients to the lifestyle questions on a self-administered questionnaire as shown in Supplementary Table 1. The patients in this group had less habitual intake of high-temperature foods and much higher intake of green/yellow vegetables and fruits. None of the patients had alcohol flushing or were in a secondhand smoke environment. These observations suggested that the patients in the VLR-group did not have commonly assumed risk factors for ESCC development.

Notably, 18 (85.7%) of the patients in this group were negative for *Helicobacter pylori*, observed by either urea breath test or *H. pylori* antibody testing. Their FSSG scores ranged from 6 to 25 points (median score: 9.6 points), and 19 patients (90.4%) scored more than 8 points (the putative cut-off points for GERD symptoms). In addition, approximately two-thirds of patients in the VLR-group complained of the symptoms of pharyngolaryngeal paresthesia, possibly related to reflux to the upper esophagus or larynx. According to their medication histories, two-thirds of the patients in the VLR-group took proton pump inhibitors (PPIs) for a perioperative period owing to GERD symptoms. Taken together, most of the patients in the VLR-group suffered from GERD symptoms and needed to take PPIs.

Table 1 Comparison of the characteristics of patients between VLR-group and R-group

	VLR-group (n = 21)	R-group (n = 491)	p value
Sex			< 0.001
Male	0 (0)	435 (88.6)	
Female	21 (100)	56 (11.4)	
Mean age ± SD (years)	67.7 ± 8.8	69.9 ± 8.6	0.264
Mean body mass index ± SD (kg/mg^2)	20.8 ± 2.6	22.3 ± 4.9	0.158
Alcohol drinking			< 0.001
Current/ex-drinker	0 (0)	446 (90.8)	
Never drinker	21 (100)	45 (9.2)	
Cigarette smoking			< 0.001
Current/ex-smoker	0 (0)	377 (76.8)	
Never smoker	21 (100)	114 (23.2)	
LVL grade			< 0.001
A	21 (100)	19 (3.9)	
B	0 (0)	275 (56.0)	
C	0 (0)	197 (40.1)	
Family history of ESCC	0 (0)	27 (5.5)	0.618
Synchronous ESCC	0 (0)	78 (15.9)	0.057
Metachronous ESCC	1 (4.8)	66 (13.4)	0.339
Previous history of cancer	2 (9.6)	188 (38.3)	0.0093
Previous history of head and neck cancer	0 (0)	68 (13.8)	0.094
Post gastrectomy	1 (4.8)	52 (10.6)	0.712
Post CRT for ESCC	0 (0)	26 (5.3)	0.617
GERD (LA-Grade)			< 0.001
Absence/Presence	5/16 (23.8/76.2)	367/84 (74.5/25.5)	
Grade A/B	16	82	
Grade C/D	0	2	
Hiatal hernia			< 0.001
Hill Grade I–I	3 (14.3)	350 (71.3)	
Hill Grade III–IV	18 (85.7)	141 (28.7)	
Atrophic gastritis			< 0.001
Closed type (C1-3)	17 (81.0)	170 (34.6)	
Open type (O1-3)	4 (19.0)	321 (65.4)	

Categorical data are presented as number (%), continuous data are presented as mean ± standard deviation

CRT chemoradiation therapy, *ESCC* esophageal squamous cell carcinoma, *GERD* gastroesophageal reflux, *LVL* lugol-voiding lesions, *R-group* risk-group, *SD* standard deviation, *VLR-group* very low-risk-group

Clinicopathological features of tumors in the VLR-group

We carefully characterized the endoscopic features of tumors obtained from the patients in the VLR-group; two clear tumor types were apparent: a linear type (n = 15) and a round type (n = 7). The linear type was defined as a lesion with a major axis that was at least twice the minor axis, and the round type was defined otherwise. We could not find linear type lesions in the R-group. Typical endoscopic features are shown in Fig. 1. Clinicopathological features of tumors in the VLR-group are shown in Table 3. Location of tumors in the middle thoracic esophagus (Mt) (73.3% vs. 28.6%; $p < 0.029$), 5–8 o'clock direction of the posterior wall (100% vs. 28.6%; $p < 0.001$), GERD (100% vs. 16.7%; $p < 0.001$), and endoscopic white coating appearance (87.5% vs. 16.7%; $p < 0.001$) were more frequently observed in the linear-type tumors than round-type tumors. Figure 2 shows representative endoscopic features of the linear-type tumors in the VLR-group. The background GERD-like inflammation extended longitudinally, and the distance of esophagitis spread about 80 mm, although the size of the tumor was only 20 mm. The tumor border was obscured, with gradual transition to esophagitis.

Table 2 Difference in tumor characteristics of lesions between the VLR group and the R-group

	VLR-group (n = 22)	R-group (n = 682)	p value
Macroscopic type			1
Depressed	22 (100)	665 (97.5)	
Protruded/Flat	0 (0)	17 (2.5)	
Location 1			0.955
Upper esophagus (Ce, Ut)	4 (18.2)	113 (16.6)	
Mid esophagus (Mt)	13 (59.1)	401 (58.8)	
Lower esophagus (Lt. Ae)	5 (22.7)	168 (24.6)	
Location 2*			0.0029
A (11–2 o'clock direction)	2 (9.1)	89 (13.6)	
B (2–5)	3 (13.6)	176 (26.9)	
C (5–8)	17 (77.3)	260 (39.8)	
D (8–11)	0 (0)	129 (19.7)	
Median tumor size (range)	19.5 (5–35)	18.0 (1–110)	0.87
Median specimen size (range)	38.0 (15–70)	31.5 (8–180)	0.17
Depth of invasion			0.0019
IN	1 (4.5)	22 (3.2)	
EP	18 (82.0)	272 (39.9)	
LPM	1 (4.5)	263 (38.6)	
MM	2 (9.0)	70 (10.3)	
SM1	0	19 (2.8)	
SM2	0 (0)	36 (5.2)	
Lymphovascular infiltration			
(+)	0 (0)	17 (2.5)	1
(−)	22 (100)	665 (97.5)	
En-bloc resection	22 (100)	675 (99.0)	1
R0 resection	22 (100)	667 (97.8)	1
Curative resection	20 (90.0)	557 (81.7)	0.399

Categorical data are presented as number (%), continuous data are presented as mean ± standard deviation. Proportions compared with chi-square

IN intraepithelial neoplasia, *EP* epithelium, *LPM* lamina propria mucosae, *MM* muscularis mucosae, *SM1* superficial submucosa (≦ 200 μm), *SM2* invading the mid submucosae (> 200 μm), *Ce* cervical esophagus, *Ut* upper thoracic esophagus, *Mt* middle thoracic esophagus, *Lt* lower thoracic esophagus, *Ae* abdominal esophagus, *VLR-group* very low-risk-group, *R-group* risk-group

*Circumferential lesions were excluded

In contrast, rounded lesions were found in the background without esophagitis, and mainly located at the lower thoracic esophagus.

Histological and genetic features of tumors in the VLR-group

Histopathological inflammation of the background mucosa (regenerative squamous epithelium, basal cell hyperplasia, and lamina propria fibrosis by hematoxylin–eosin staining) was observed in 13 out of 15 (86.7%) lesions of the linear-type tumors, whereas only 2 out of 7 (28.6%) lesions in the round type showed this feature (p = 0.014).

Figure 3 shows the pathological findings in a representative case of the linear-type tumor in the VLR-group. Histologically, in these lesions, features such as atypical nuclei were limited to the basal and parabasal layers and presented typical pathological findings of chronic inflammation around the tumor.

The *TP53* gene is the most common genetic mutation in cancers, including ESCC. This mutation can lead to increased *TP53* expression in nuclei. We performed p53 immunostaining of tumors for all cases in the VLR-group. Immunostaining of the tumor was strongly positive for p53 in the nuclei of the cancer cells in the basal and parabasal layer in 60% (9/15) of the lesions of the linear type and

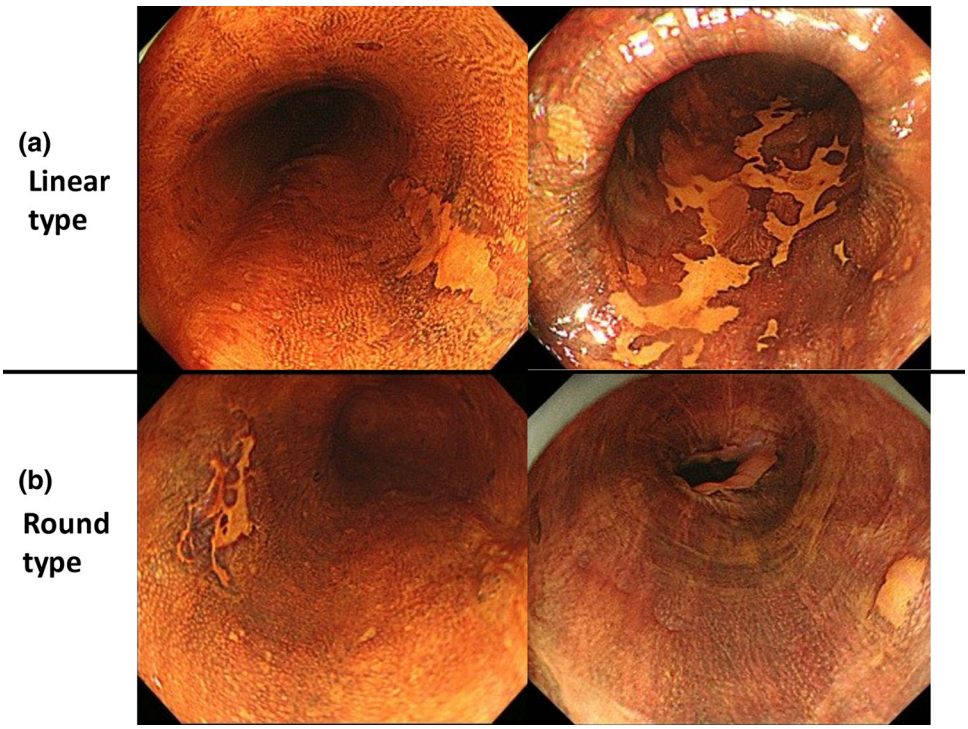

Fig. 1 Classification of endoscopic features of tumors in the very low-risk (VLR)-group. A linear-type tumor was defined as a lesion with a major axis at least twice the minor axis (**a**), and a round type was defined otherwise (**b**)

Table 3 Clinicopathological differences in tumor characteristics between the linear and rounded lesions in the VLR-group

	Linear type (n = 15)	Round type (n = 7)	p value*
Location 1			0.029
Upper esophagus (Ce/Ut)	3 (20)	1 (14.3)	
Mid esophagus (Mt)	11 (73.3)	2 (28.6)	
Lower esophagus (Lt/Ae)	1 (6.7)	4 (57.1)	
Location 2			0.0008
A (11–2 o'clock direction)	0 (0)	2 (28.6)	
B (2–5)	0 (0)	3 (42.9)	
C (5–8)	15 (100)	2 (28.6)	
D (8–11)	0 (0)	0 (71.4)	
Pathological depth			0.041
IN	1 (6.7)	0 (0)	
EP	14 (93.3)	4 (57.1)	
LPM	0 (0)	1 (14.3)	
MM	0 (0)	2 (28.6)	
SM1	0 (0)	0 (0)	
GERD	15 (100)	1 (16.7)	< 0.001
Pathological inflammation of background mucosa	13 (86.7)	2 (28.6)	0.014
White coating appearance	14 (93.3)	2 (28.6)	0.004

Categorical data are presented as number (%), proportions compared with chi-square

Ce cervical esophagus, *Ut* upper thoracic esophagus, *Mt* middle thoracic esophagus, *Lt* lower thoracic esophagus, *Ae* abdominal esophagus, *IN* intraepithelial neoplasia, *EP* epithelium, *LPM* lamina propria mucosae, *MM* muscularis mucosae, *SM1* superficial submucosa (≦ 200 μm), *SM2* invading the mid submucosae (> 200 μm), *GERD* gastroesophageal reflux, *VLR-group* very low-risk-group

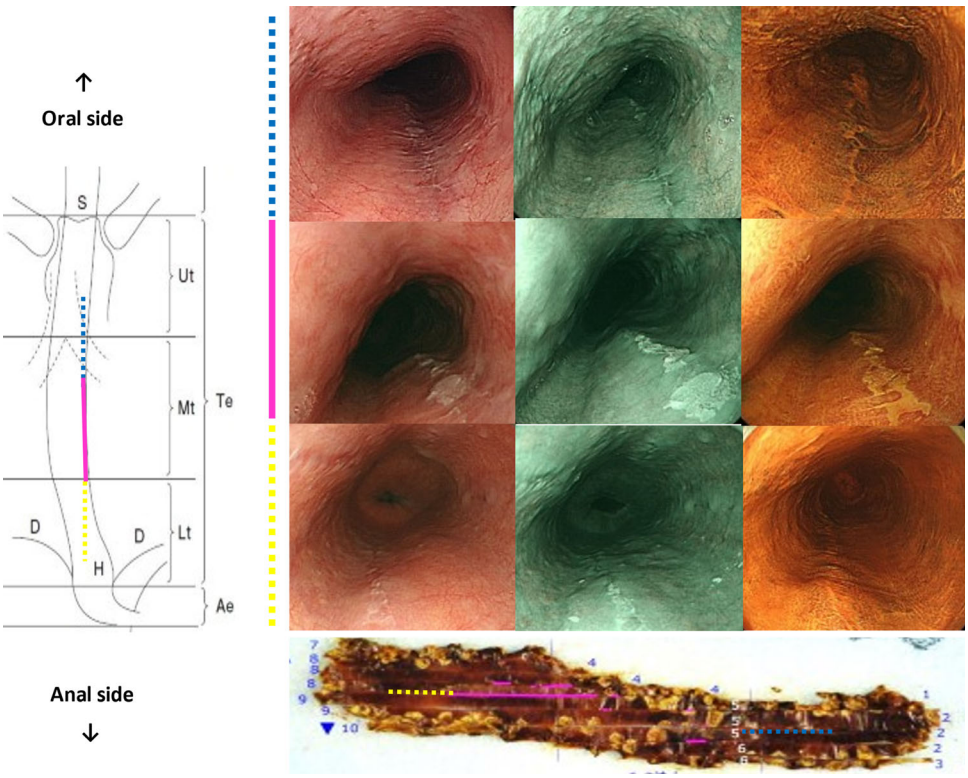

Fig. 2 Representative endoscopic features of linear type lesions in the very low-risk (VLR)-group. The background GERD-like inflammation extended longitudinally, and the distance of esophagitis spread about 80 mm, whereas the size of tumor was only 20 mm (pink line). Diagnosis of tumor depth by ME-NBI (magnifying endoscopy with narrow-band imaging) was difficult due to the appearance of a white coating of esophagitis. Due to the unclear boundary between inflammation and tumor, the tumor resection included the entire Lugol-unstained lesion. *Ut* upper thoracic esophagus, *Mt* middle thoracic esophagus, *Lt* lower thoracic esophagus

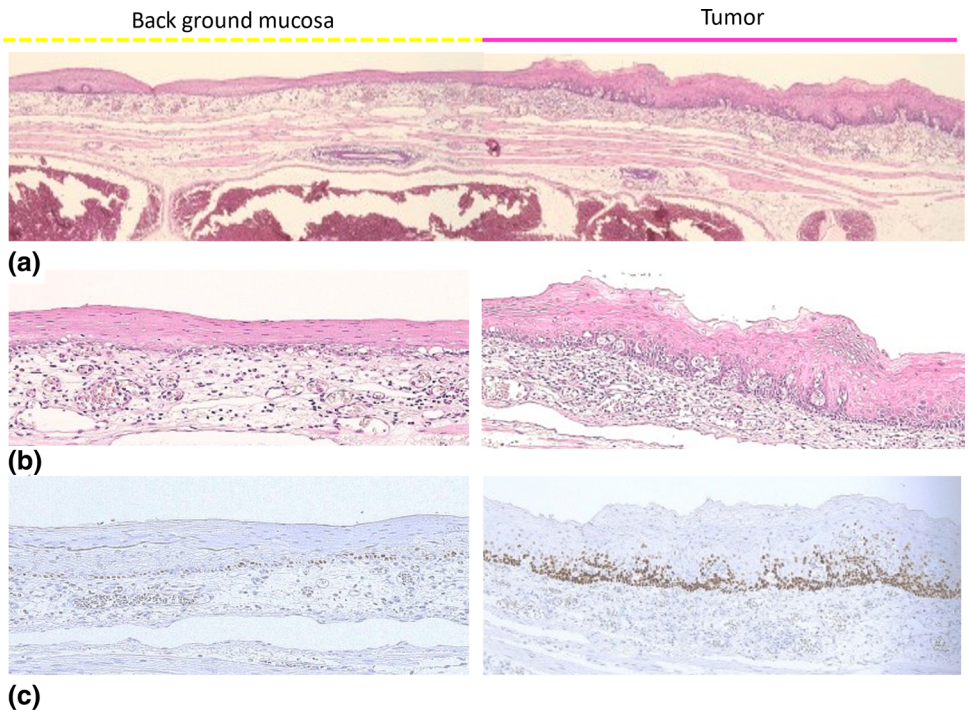

Fig. 3 **a** Pathological findings of a representative case of a linear-type tumor in the very low-risk (VLR)-group. **b** Histologically, linear type lesions exhibit atypical nuclei limited to the basal and parabasal layer and present with typical pathological findings of chronic inflammation around the tumor. In linear-type lesions, regenerative squamous epithelium is present on the background mucosa because of esophagitis. **c** The p53 immunostaining of tumors was strongly positive in the nuclei of cells in the basal and parabasal layer

57.1% (4/7) of the lesions of the round type in the VLR-group.

In addition, we performed cancer-related gene target sequencing analysis to investigate potential relationships and differences in ESCC somatic mutations especially in patients in the VLR-group (Fig. 4; Supplementary Table 2). A total of 125–600 ng of genomic DNA per sample was extracted for sequencing. The quality for all the lesions in the library for which NGS was performed cleared the cut-off values. NGS analysis was performed on

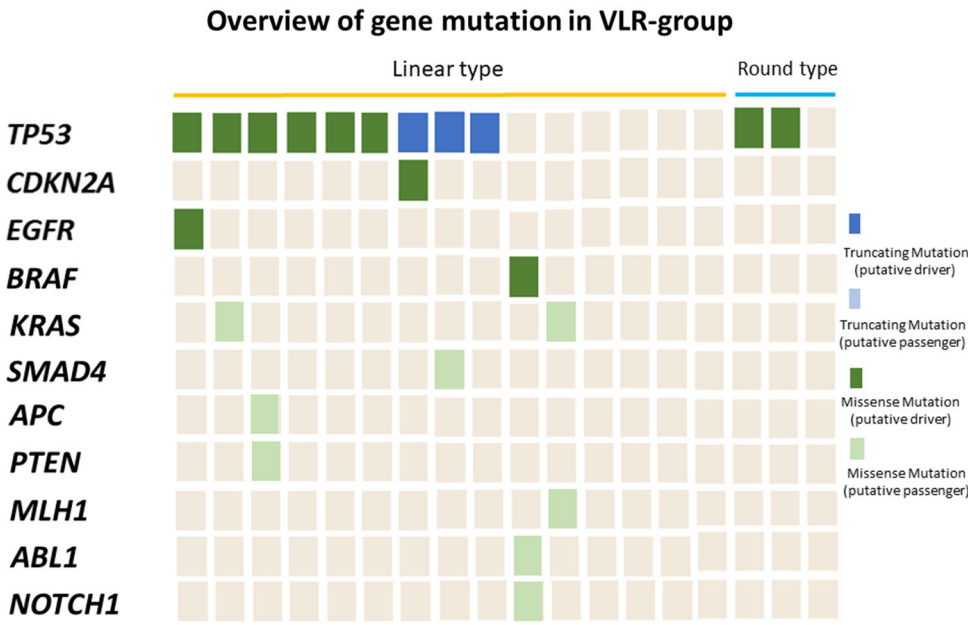

Fig. 4 *TP53* was the most frequently mutated gene in 9/15 (60.0%) of the linear-type and 2/3 (66.7%) of the round-type lesions in the VLR-group

15 lesions of the linear type and 3 lesions of the round type from the VLR-group. The CHP v2 NGS analysis revealed some putative driver mutations in 11/15 lesions of the linear type and 2/3 lesions of the round type in the VLR-group (Fig. 4). Notably, in our cohort, *TP53* was most frequently mutated gene, found in 9/15 (60%) lesions of the linear type and 2/3 (66.7%) of the round-type lesions in the VLR-group. Immunohistochemistry revealed that 9/11 (81.8%) lesions with NGS-detected *TP*53 mutations stained positively for p53 (Fig. 4; Supplementary Fig. 2).

Discussion

In addition to drinking alcohol and smoking, LVLs are a well-known risk for ESCC carcinogenesis [9, 10]. In clinical practice, we are sometimes faced with ESCC cases that do not have these major causative risk factors; reports about such cases have been very few. Kuwabara et al. reported the clinical characteristics of ESCC without LVLs in the background esophagus [20]. In their study, the comparison only included groupings with or without an LVL. We believe that this classification alone is not sufficient for accurate risk assessment for ESCC because the carcinogenic process is reported to be multi-factorial. Katada et al. [9] reported that the grade of LVL is a useful predictor of the risk for metachronous multiple ESCCs. However, they also included patients with an alcohol and/or smoking history, which are representative risk factors for ESCC, in the LVL-negative group. Therefore, LVL-negative does not equate with the low-risk patients in terms of developing ESCC. The definition of low-risk factors in the present study is more stringent than in previous studies. Shigaki et al. described the clinicopathological features of 4.3% (30/691) of patients with ESCC who underwent esophagectomy; the subjects were never-smokers and never-drinkers and females of advanced age with well-differentiated tumor histology and a family history of esophageal cancer [7]. The significantly higher proportion of the female sex in our study is the same as in the previous report; however, advanced age and family history of esophageal cancer were not presented by the patients in our cohort, for reasons that remain unclear. As this report included all pathological stages and only three cases of pT0 and nine cases of pT1, details of early stage lesions were not evaluated. In our present study, all of the tumors in the VLR-group were pT0 and all exhibited naturally resulted well-differentiated lesions. Only 1 out of 21 cases in the VLR-group suffered from metachronous ESCC. The period from the first ESD to the detection of a metachronous secondary lesion was about 3 years. The patient continued to take PPIs after ESD but showed PPI-resistant refractory GERD. The endoscopic findings during follow-up showed four radial lines due to GERD; 3 years later, the neoplastic lesion appeared on another GERD line. We speculate that neoplastic change had already occurred at the time of primary treatment, and the possibility that we could not detect it because of an infinitesimal change cannot be ignored. However, because the period until redetection was as long as 3 years, we considered this to be a metachronous lesion derived from GER.

As new knowledge, the present study suggests a relationship between GERD and the occurrence of ESCC in the VLR-group, possibly due to significantly higher prevalence

of hiatal hernia and negativity for *H. pylori*. Chronic reflux esophagitis is typically the predominant causative factor for the development of Barrett's esophagus and its progression to EAC [6]. However, a few studies reported the relationship between ESCC and the GERD condition [19, 20]. Our findings are consistent with a prior meta-analysis that demonstrated a role for GERD in laryngopharyngeal carcinogenesis [21]. Based on responses on the FSSG questionnaire [18], 90% of patients in the VLR-group scored high, suggesting that they had symptoms of GERD.

The association between a negative *H. pylori* status and reflux esophagitis has been epidemiologically demonstrated, given the decreased gastric acid secretion by gastric atrophy in *H. pylori*-infected patients [22]. Furthermore, osteoporosis often leads to spinal kyphosis, which has been implicated as a contributor to the increased frequency of GERD [23, 24]. The significances of these conditions are supported by the relationship between ESCC in our VLR-group and GERD. In addition, we showed a unique endoscopic feature with morphology similar to linear esophagitis, mainly located on the posterior wall of the middle thoracic esophagus, without LVL. Lying down after eating can result in duodenal fluid, which contains bile acids, remaining in the stomach and collecting in the fornix on the dorsal side. When transient lower esophageal sphincter relaxations occur in this state, the esophagogastric junction and the lower to middle esophagus are exposed to the acidic digestive fluid that might accumulate on the posterior wall of the esophagus [28].

The specific endoscopic appearance of linear-type ESCC has been shown in a few reports [19, 20], but there has been no report regarding its pathological verification.

Previous studies assumed a GERD-related carcinogenesis association only by endoscopic macroscopic features. In the present study, as our most advantageous point, a pathological evaluation of the association with GERD was made. This endoscopic characteristic of lesions of the linear type was difficult to discriminate endoscopically; whether the apparent white coating was a feature of inflammation or neoplasia was not clear, and it was challenging to confirm the range of the tumor with the existence of GERD in the background. Nevertheless, based on assessments using hematoxylin–eosin staining, our findings emphasized that histopathological findings of GERD (regenerative squamous epithelium, basal cell hyperplasia, and lamina propria fibrosis on background mucosa) were frequently observed on the background mucosa of the linear type of SCC [25]. This breakthrough finding suggests that inflammation due to GERD may be the background of ESCC carcinogenesis.

Several basic research studies have also evaluated the association between ESCC and acid reflux due to both gastric acid and non-acid reflux of duodenal contents [26–31]. Miwa et al. used duodenoesophageal reflux rat models to demonstrate that ESCC developed in locations that are distant from the anastomosis, as compared with that of EAC [26, 27]. These findings suggested that small and continuous quantities of reflux contents might be associated with the development of ESCC. Whether chronic reflux induces EAC or ESCC may depend on the volume and composition of the bile acids in the reflux agents. Mukaisho et al. developed several rat models of esophageal reflux, surgically induced by re-routing bile acid-containing duodenal contents back into the esophagus. They showed that continuous exposure of the esophagus to bile acid promoted ESCC progression. These basic analyses support that most cases of ESCC in the VLR-group tend to occur in the middle esophagus rather than in the lower esophagus [26–31]. In humans, a few studies using the 24-h multichannel intraluminal impedance-pH (MII-pH) monitoring test revealed the clinical significance of GER [32, 33]. Specifically, they demonstrated a high frequency of GER, especially non-acid reflux, which may be an important factor in the development of ESCC. Whether we can derive similar results from MII-pH monitoring in the VLR-group is a topic for future studies.

Several whole-exome sequencing studies have revealed the landscape of driver genes, as well as somatically disrupted pathways in ESCC. Mutations of *TP53*, *CDKN2A*, and *PIK3CA* during amplification are common in ESCC. A high frequency of *TP53* mutations in ESCC (including early ESCC) has been reported since the 1990s [34–37]. Clinically, smoking is associated with the occurrence of *TP53* mutations in early lesions [34–39], and the high frequency of *TP53* mutations in inflammatory carcinomas is generally known [40].

Recently, whole genome or exome NGS of advanced ESCC confirmed that *TP53* is the most frequently mutated gene; however, most NGS investigations did not include risk stratification. In our study, ESCC in the VLR-group involved typical clinical features such as gastroesophageal reflux. Therefore, these tumors may have some specific driver gene mutations or somatically disrupted pathways. We examined NGS data for 18 lesions in the VLR-group. As stated above, previous studies have already shown that mutations in *TP53, CDKN2A*, and *PIK3CA* are common in early esophageal squamous neoplasia; therefore, we did not analyze lesions of the R-group as a control [35–39]. We identified *TP53* as the most frequently mutated gene in the linear-type lesions in the VLR-group (9/15 cases, 60%). This finding is similar to previous reports on ESCC [35–39], suggesting that although ESCC of the linear-type lesions in the VLR-group presented features distinct from clinical and endoscopic perspectives, the major gene mutation found in ESCC from GERD was of the same

genetic pathway as that of the classical ESCC of the R-group. No other remarkable gene mutation was found.

Interestingly, in highly inflammatory cases of the linear lesion, a low frequency of *TP53* mutation (allele frequency 1–4%) was observed also in the background mucosa.

Regarding the etiology of the round-type lesions, we assume that they are sporadic and of de novo occurrence because the endoscopic and pathological features, including the absence of inflammatory changes in the background, are clearly different from those of linear-type lesions. However, we could not identify any difference in the genetic analysis between the two groups; thus, this is beyond expectations.

In the present study, we focused only on genomic mutations. On the other hand, Urabe et al. and Liu et al. recently revealed the differences in copy number variation in early ESCC [38, 39]. Therefore, we believe that additional genetic alteration (copy number and structural variation) and epigenetic analysis are necessary in the future.

In summary, our data show no significant differences in genetic mutations in esophageal cancer with or without risk for the development of ESCC. There are several limitations in this study. First, this is a retrospective study conducted at a single institution. Second, our sample size was small. Third, the 24-h MII-pH monitoring was not conducted for evaluation of GER. Fourth, NGS was performed using a target gene panel focused on 50 cancer-related genes. Thus, further genetic analysis is needed to elucidate the origin of the VLR-group tumors.

Conclusion

Usually, GER is the cause of EAC with Barrett's esophagus; however, we found clinicopathological features of ESCC in patients without major causative factors, who had clear GERD symptoms. In these cases, a different cause of carcinogenesis was assumed, although our genetic investigation could not reveal other pertinent associations. Further research is needed to fully describe these cases in the future.

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Author contributions KH and TF contributed to the conception and design of the study. KH, TF, YO, AS, CS, RI, MN, RK, MM, and MS contributed to the acquisition of data. KH, TF, and MS contributed to the analysis and interpretation of data. KH and TF contributed to the drafting of the article. KH and SM contributed to the critical revision of the article. KH and TF contributed to statistical analysis. KH and SM approved critical revision of the article for important intellectual content and the final draft of the article. All authors listed have contributed substantially to the design, data collection and analysis, and editing of the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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論文目録

Ⅰ　主論文

Potential roles of gastroesophageal reflux in patients with superficial esophageal squamous cell carcinoma without major causative risk factors

Takehide Fukuchi, Kingo Hirasawa, Chiko Sato, Makomo Makazu, Hiroaki Kaneko, Ryosuke Kobayashi, Masafumi Nishio, Ryosuke Ikeda, Atsushi Sawada, Yuichiro Ozeki, Makoto Sugimori, Yoshiaki Inayama, Yoko Tateishi, Shin Maeda. J Gastroenterol 2021.

Ⅱ　副論文

Ⅲ　参考論文

Factors infuencing interruption of colorectal endoscopic submucosal dissection

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