Prognostic biomarker study in patients with clinical stage I esophageal squamous cell carcinoma: JCOG0502-A1

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Abstract
We undertook genomic analyses of Japanese patients with stage I esophageal squamous cell carcinoma (ESCC) to investigate the frequency of genomic alterations and the association with survival outcomes. Biomarker analysis was carried out for patients

Abbreviations: amp, amplification; CI, confidence interval; CNA, copy number alteration; CNV, copy number variant; CRT, chemoradiotherapy; CTTN, cortactin; ESCC, esophageal squamous cell carcinoma; FFPE, formalin-fixed, paraffin-embedded; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; HCC, hepatocellular carcinoma; HR, hazard ratio; ICI, immune checkpoint inhibitor; indel, insertions/deletion; JCOG, Japan Clinical Oncology Group; MC, mononuclear cell; Mut/Mb, mutations per megabase; OS, overall survival; PFS, progression-free survival; SNV, single nucleotide variant; TMB, tumor mutational burden; WES, whole-exome sequencing.
with clinical stage T1bN0M0 ESCC enrolled in JCOG0502 (UMIN000000551). Whole-exome sequencing (WES) was performed using DNA extracted from formalin-fixed, paraffin-embedded tissue of ESCC and normal tissue or blood sample. Single nucleotide variants (SNVs), insertions/deletions (indels), and copy number alterations (CNAs) were identified. We then evaluated the associations between each gene alteration with a frequency of 10% or more and progression-free survival (PFS) using a Cox regression model. We controlled for family-wise errors at 0.05 using the Bonferroni method. Among the 379 patients who were enrolled in JCOG0502, 127 patients were successfully analyzed using WES. The median patient age was 63 years (interquartile range, 57–67 years), and 78.0% of the patients ultimately underwent surgery. The 3-year PFS probability was 76.3%. We detected 20 genes with SNVs, indels, or amplifications with a frequency of 10% or more. Genomic alterations in FGF19 showed the strongest association with PFS with a borderline level of statistical significance of P = .00252 (Bonferroni-adjusted significance level is .0025). Genomic alterations in FGF4, MYEOV, CTTN, and ORAOV1 showed a marginal association with PFS (P < .05). These genomic alterations were all CNAs at chromosome 11q13.3. We have identified new genomic alterations associated with the poor efficacy of ESCC (T1bN0M0). These findings open avenues for the development of new potential treatments for patients with ESCC.

**KEYWORDS**

esophageal squamous cell carcinoma, prognostic factor, stage I, tumor mutation burden, whole-exome sequencing

1 | INTRODUCTION

Esophageal cancer is the sixth leading cause of cancer death worldwide. Squamous cell carcinoma and adenocarcinoma are the main histological types. Esophageal squamous cell carcinoma accounts for the majority of the incidence of esophageal cancer in Asia, including China and Japan. Epidemiological studies have reported that the consumption of alcoholic beverages, tobacco smoking, and hot food and beverages could be risk factors for ESCC. Specific variants in the aldehyde dehydrogenase 2 family gene (ALDH2) and the acetaldehyde dehydrogenase gene (ADH1B) are also associated with a strong risk of ESCC. In addition, as ESCC develops through a multistep process beginning with dysplasia and progresses through carcinoma in situ to invasive carcinoma, the involvement of genomic alterations in the development of ESCC has been recognized. Previous reports have shown somatic mutations and CNVs in ESCC as follows: TP53, CCND1, PIK3CA, NOTCH1, ZNF750, FAM135B, FAT1, and FAT2.

At present, esophagectomy is the standard therapy for T1bN0 ESCC. We previously reported that definitive chemoradiotherapy showed a noninferior outcome, compared with an esophagectomy, in terms of OS in the JCOG0502 trial. After recurrence, however, the treatment options were limited and the prognosis was poor, even though the initial diagnosis had been T1bN0M0. The mean survival time of patients with recurrence after esophagectomy was reported to be 20 months. Several clinicopathologic features have been associated with recurrence or metastatic risk in T1bN0M0 ESCC, including tumor size, tumor location, tumor invasion depth, angiolymphatic invasion and tumor thickness, and complete negative aberrant p53 expression. In addition, previous reports have shown that genomic alterations were associated with prognosis. Shigaki et al reported that patients with PIK3CA mutations in exons 9 and/or 20 showed significantly better disease-free survival and OS outcomes than patients with the PIK3CA WT. Sawada et al reported that EP300 and TET2 mutations were associated with a poor prognosis. However, these reports evaluated patients with various background factors and disease stages. Few reports have examined genetic alterations in only T1bN0M0 ESCC or have investigated the association between genomic alterations and survival outcomes. Information on genomic alterations with prognostic or predictive value could be useful not only for the selection of additional treatments based on the risk of recurrence, but may suggest novel therapeutic options for patients with ESCC.

Therefore, we analyzed genomic alterations using WES and investigated the association between these genomic alterations and prognosis in T1bN0M0 ESCC patients who enrolled in prospective clinical trial JCOG0502.

2 | MATERIALS AND METHODS

2.1 Study design and patient selection

JCOG0502A1 was a biomarker study using biosamples obtained from patients who enrolled in JCOG0502. JCOG0502 was a clinical trial comparing esophagectomy with definitive chemoradiotherapy for T1bN0M0 esophageal cancers and included both randomized
arms and patient-preference arms. The key eligibility criteria for JCOG0502 were as follows: age between 20 and 75 years, diagnosis of histologically proven clinical stage IA (T1bN0) squamous cell carcinoma, adenosquamous cell carcinoma, or basaloid cell carcinoma in the thoracic esophagus (American Joint Committee on Cancer Staging Manual, 7th edition), and performance status of 0-1 according to the ECOG performance status scale. The major exclusion criteria were as follows: double primary cancer, uncontrolled diabetes, recent myocardial infarction, unstable angina, and severe complications.

Written informed consent for this ancillary study was directly obtained from the patients in principle. However, if the patients were dead or lost to follow-up, the use of samples from these patients was approved by the Institutional Review Board, which privileged the opt-out for the patients, according to the Ethical Guidelines for Epidemiological Research issued by the Ministry of Education, Culture, Sports, Science and Technology and the Ministry of Health, Labour and Welfare of Japan.

The study protocol was approved by the JCOG Protocol Review Committee and by the review boards of all the participating institutions.

## 2.2 Sample collection

Tumor samples and histologically normal tissues or blood samples were obtained from 138 patients who had participated in JCOG0502A1. Tumor samples were collected from the surgical specimens or from biopsy samples obtained before surgery or definitive chemoradiotherapy.

Tissue DNAs were extracted from sliced FFPE biopsy specimens and surgical tissue samples using the QIAamp DNA FFPE Tissue Kit (Qiagen), and DNAs were extracted from blood using the QIAamp DNA Blood Kit (Qiagen).

Blood samples were collected and centrifuged according to the manufacturer’s instructions and were used to separate the MCs; the separated plasma was frozen and stored at −80°C in the JCOG-Biobank Japan Biorepository (JCOG BioBank). The DNA of MCs was also extracted from PBMC samples obtained from each patient and stored at 4°C in the JCOG BioBank. Some of the stored MC DNA was used for this study.

## 2.3 Whole-exome sequencing and mutation calls

Extracted FFPE DNAs were evaluated using the PicoGreen dsDNA assay (Thermo Fisher Scientific) and the TaqMan Copy Number Reference qPCR assay (Thermo Fisher Scientific). The libraries were prepared with the KAPA HyperPrep Kit (Kapa Biosystems) using 10-50 ng FFPE DNAs according to the manufacturer’s protocol with some modifications after the fragmentation process (60 s × 3 cycles) using COVARIS. The exome capture was undertaken with SureSelect Human All Exon V5 (Agilent Technologies), and sequencing was carried out with Illumina HiSeq2500 SBS V4 (Illumina). Sequence reads were aligned to the human reference GRCh37 using bwa-0.7, and the sequencing average coverage was 116x for the tumor-specimen DNAs and 92x for normal tissue DNAs after duplication removal. Somatic mutation calls were performed using Genomon 2.6.1 ([https://genomon.readthedocs.io/ja/latest/](https://genomon.readthedocs.io/ja/latest/)). For reliable calls, we further selected mutations with variant allele frequencies of 10% or more.

## 2.4 Copy number analysis

Copy number alterations were called using cisCall (https://www.ciscall.org/), which is a calling tool specialized for FFPE samples. During preprocessing, we removed the sequence adapters using cutadapt (https://cutadapt.readthedocs.io/).[16] Mapping and the removal of duplications were performed internally using cisCall. From among the CNAs detected using cisCall, we further selected CNAs with log R ratios of |1.5| or more and that covered at least 80% and 70% of a gene for amplifications and losses as stringent calls, respectively. In the sensitivity analysis, we used a looser criteria of a log R ratio of |1.0| or more and amplification and loss coverages of at least 60%.

## 2.5 Tumor mutational burden

Tumor mutational burden was defined as the number of SNVs and indels per megabase of WES.

## 2.6 Statistical methods

The probability of PFS was estimated using the Kaplan-Meier method, and Greenwood’s formula was used to calculate the 95% CIs. A Cox regression model that included age (≤64 years, ≥65 years) and tumor size (≤4 cm, >4 cm) was used to estimate HRs and 95% CIs, P values for each gene with an alteration frequency of at least 10%, and TMB. Treatment arm was not included in the Cox regression model as no association was observed in this population (univariate HR = 1.03; 95% CI, 0.49-2.16). Gene alteration was defined as the presence of at least one SNV, indel, or CNA. Family-wise error for the gene-level analysis was controlled at 0.05 using the Bonferroni method.

## 3 RESULTS

### 3.1 Patient characteristics and PFS time in this study

Among the 379 patients who were enrolled in JCOG0502, WES was successfully carried out for 127 patients in this study, JCOG0502A1 (Figure 1). The clinicopathologic characteristics of
all 379 patients who were enrolled in JCOG0502 and the 127 patients who successfully underwent WES in this study are listed in Table S1. In this study, 99 patients (78%) underwent surgery (surgery group), and 28 patients (22%) received definitive CRT (CRT group). The proportion of patients in the surgery group in this study tended to be larger than the proportion of patients who were enrolled in JCOG0502. The 3-year and 5-year PFS of all patients included in JCOG0502 and the 127 patients included in this study are summarized in Table S2.

3.2 Detection of genomic alterations

In the 127 ESCC samples, a total of 13 764 mutations, including 12 963 SNVs and 801 indels, were detected using WES. These SNVs and indels contained 5126 nonsilent SNVs in 3990 genes and 206 indels in 193 genes. The mean number of mutations was 108.4 per sample. We found that 60.6% (77/127) of ESCC had CNAs at the chromosome arm level. A total of 3666 amplifications among 2054 genes and 2081 losses among 1232 genes were detected across the 127 ESCC samples.

The most frequent genomic alterations were found in TP53 (mutated in 35% of our cohort), followed by HIST2H2AA3 (CNAs [amp] in 19% and CNAs [loss] in 2%), HIST2H3A (CNAs [amp] in 19% and CNAs [loss] in 2%), HIST2H4A (CNAs [amp] in 17% and CNAs [loss] in 2%), ORAOV1 (CNAs [amp] in 17%), FADD (CNAs [amp] in 15%), CCND1 (CNAs [amp] in 14%), PPFIA1 (CNAs [amp] in 13% and mutated in 1%), FGF19 (CNAs [amp] in 12%), LGALS7 (CNAs [amp] in 12%), MYEOV (CNAs [amp] in 12%), FGF4 (CNAs [amp] in 11%), LGALS7B (CNAs [amp] in 11%), MUC16 (mutated in 10%, CNAs [amp] in 1%), NKX2-4 (CNAs [amp] in 8% and CNAs [loss] in 3%), TGF2LY (mutated in 1%, CNAs [amp] in 2%, CNAs [loss] in 8%), ZNF750 (mutated in 11%), and ANO1 (CNAs [amp] in 10%), and CTTN (CNAs [amp] in 10%) (Figure 2).

3.3 Associations between genomic alterations and PFS

We detected 20 genes with SNVs, indels, or CNAs (amp) with a frequency of at least 10% and analyzed the associations between these genomic alterations and PFS (Table 1). The Bonferroni method was applied to correct for the family-wise error, resulting in a significance level at .0025 (= .05/20). Genomic alterations in FGF19 showed the strongest association with PFS with borderline level of statistical significance (P = .00252). Marginal associations with PFS (P < .05) were observed for the following genomic alterations: FGF4 (P = .0053), MYEOV (P = .0238), CTTN (P = .0296), and ORAOV1 (P = .0420). The Kaplan-Meier curves for these genomic alterations are shown in Figure 3. These genetic alterations are located within the chromosomal region 11q13.3 (Figure 4). We further investigated the association between these five genomic alterations and patient characteristics (Table S3). Genomic alterations in FGF19 and CTTN were detected significantly more often in patients aged 65 years or older, compared with those younger than 65 years. These five genomic alterations were also detected more frequently in the CRT group than in the surgery group. Therefore, we undertook a sensitivity analysis using a Cox regression model that included treatment arm (surgery, CRT), as well as age (≤64 years, >65 years) and tumor size (≤4 cm, >4 cm). These five genomic alterations showed similar associations with PFS as follows: FGF19 (P = .0017), FGF4 (P = .0052), MYEOV (P = .0158), CTTN (P = .0244), and ORAOV1 (P = .0270). In addition, we analyzed the association between these genomic alterations and PFS in each treatment group (Table S4).

In a sensitivity analysis using data with looser criteria for CNA, we observed 125 genes with an alteration frequency of 10% or more. Genomic alterations in FGF19 and FGF4 were significantly associated with PFS (P = .00015 and .00006, respectively), and CTTN had a marginal association with PFS (P = .00694).
Median TMB was 0.84 mut/Mb (interquartile range, 0.31-2.59 mut/Mb). A TMB of 10 mut/Mb or higher was observed in four patients (3.1%). To explore the trend associations between TMB and PFS, we divided the patients into three groups: TMB <1 mut/Mb (n = 66), 1 mut/Mb ≤ TMB <3 mut/Mb (n = 34), and TMB ≥3 mut/Mb (n = 27). The PFS of patients in the 1 mut/Mb ≤ TMB <3 mut/Mb category and TMB ≥3 mut/Mb category was not significantly different from that of patients in the TMB <1 mut/Mb category (HR, 1.25; 95% CI, 0.63-2.46; and HR, 0.85; 95% CI, 0.37-1.93, respectively) (Figure 5).

We examined genetic alterations in T1bN0M0 ESCC using WES and analyzed the association between genetic alterations and PFS, not OS, because the prognosis of patients with T1bN0M0 ESCC is so good, and only 10% of the patients had cancer-specific death. Our results showed that CNV amplification in FGF19, FGF4, MYEOV, CTTN, and ORAOV1 were associated with a poorer PFS in T1bN0M0 ESCC patients. Interestingly, these genetic alterations were located within chromosomal region 11q13.3. To the best of our knowledge, this study is the first to analyze the association between genomic alterations and high-quality clinical data from a clinical trial for efficacy and safety in ESCC.
**TABLE 1** Association between genomic alterations and progression-free survival in 127 patients with T1bN0M0 esophageal squamous cell carcinoma

| Gene    | N   | HR^a | 95% CI          | P value |
|---------|-----|------|-----------------|---------|
| FGF19   | 15  | 3.10 | 1.49-6.46       | .0025   |
| FGF4    | 14  | 2.94 | 1.38-6.27       | .0053   |
| MYEOV   | 15  | 2.38 | 1.12-5.05       | .0238   |
| CTTN    | 13  | 2.38 | 1.09-5.18       | .0296   |
| ORAOV1  | 21  | 2.06 | 1.03-4.14       | .042    |
| ANO1    | 13  | 1.86 | 0.81-4.25       | .1406   |
| GABARAP | 13  | 1.82 | 0.73-4.57       | .2004   |
| CCND1   | 18  | 1.79 | 0.84-3.79       | .1306   |
| FADD    | 19  | 1.74 | 0.83-3.67       | .143    |
| PPFIA1  | 18  | 1.72 | 0.82-3.62       | .1535   |
| TTN     | 13  | 1.16 | 0.45-2.97       | .758    |
| ZNF750  | 14  | 1.03 | 0.40-2.65       | .9445   |
| TP53    | 44  | 0.79 | 0.41-1.52       | .474    |
| MUC16   | 14  | 0.75 | 0.27-2.10       | .5799   |
| LGALS7B | 14  | 0.56 | 0.17-1.82       | .3384   |
| LGALS7  | 15  | 0.50 | 0.16-1.63       | .254    |
| HISTH2AA3 | 26  | 0.50 | 0.20-1.29       | .1537   |
| HISTH23A| 26  | 0.50 | 0.20-1.29       | .1537   |
| HISTH24A| 23  | 0.43 | 0.15-1.22       | .114    |
| HISTH2BE | 13  | 0.34 | 0.08-1.40       | .1351   |

Abbreviation: CI, confidence interval.
^aHazard ratios (HRs) were estimated using the Cox model with patient and tumor size included as variables.

Fibroblast growth factor 19 is a member of the hormone-like FGF family. Normal FGF19 regulates bile acid synthesis, glucose and lipid metabolism, and gallbladder volume. Fibroblast growth factor 19 induces the expression of profibrogenic and pro-tumorigenic connective tissue growth factor in hepatocytes; thus, FGF19 contributes to hepatocarcinogenesis and leads to the development of HCC. In addition, FGF19 has been shown to induce epithelial-mesenchymal transition, which is associated with the invasion and metastasis of tumor cells, through the FGF19/FGFR4 signaling pathway in HCC and colorectal cancer cells. FGF19 amplification and overexpression are reportedly associated with a poorer prognosis for several tumor types. However, there are few reports on the predictive role of FGF19 in ESCC. Our study indicated that FGF19 amplification was the alteration most strongly associated with a poor prognosis in T1bN0 ESCC. A previous report showed that an anti-FGF19 mAb that selectively blocks the interaction between FGF19 and FGFR4 inhibited the growth of colon tumor xenografts in vivo and effectively neutralized circulating FGF19 to prevent HCC tumor formation in FGF19 transgenic mice. These findings suggest that the inactivation of FGF19 might be a therapeutic target for ESCC.

Fibroblast growth factor 4 plays an important role in the growth and differentiation of human embryonic stem cells. FGFR4 amplification and overexpression have also been reported in various tumors. FGFR3/FGF4 amplification was shown to mediate the overexpression of FGFR3/FGFR4 proteins in HCC, and FGF4 was partially involved in the sensitivity to sorafenib in an in vivo study. A previous report found that high FGF4 expression was associated with advanced stage, aggressive histological subtype, and poorer prognosis in patients with ovarian cancer. Huang et al reported that FGF4 amplification was observed using FISH in 52.8% of patients with early and advanced ESCC. They reported that FGF4 amplification was an independent prognostic factor and might have the potential for the progression of ESCC clinical stage. We showed that CNA amplification in FGF4 was associated with a poor PFS in T1bN0 ESCC patients, although the frequency of patients with CNA amplification in FGF4 was 11%.

Contactin binds and activates the actin-related protein complex, regulating the formation of dynamic cortical actin-associated structures, and CTTN is known to play a critical role in tumor cell motility and invasion. An in vivo assay showed that the inhibition of CTTN expression decreased the tumor growth and lung metastasis of ESCC cells. A previous report showed a significant association between the overexpression of CTTN and a shorter disease-specific survival period in ESCC patients with both early and advanced pathological stages. Although the role of CTTN in clinical treatments for ESCC patients has not yet been elucidated, we found that CNA amplification in CTTN was associated with poor PFS in T1bN0 ESCC patients. MYEOV has been reported to be overexpressed and to contribute to tumorigenesis and a poorer prognosis in several cancers, including ESCC. MYEOV enhances tumorigenicity and tumor growth and is associated with a poorly differentiated tumor histology in ESCC through proline metabolism and reactive oxygen species production. Previous reports have shown that ORAOV1 amplification or overexpression is associated with poor prognosis. Our results were consistent with these previous reports.

The 11q13 region could be central to ESCC development. The 11q13 region contains several genes including FGFR3, FGFR4, CCND1, MYEOV, FGF19, and SHANK2. The amplification of 11q13 has been reported in tumors, including ESCC, and several studies have shown associations with metastasis and poor survival. A previous report showed that CCND1 and CCND1 are frequently amplified in ESCC and head and neck squamous cell carcinoma. Ying et al reported that CCND1 amplification/overexpression was significantly associated with lymph node metastasis of ESCC. Of 18 patients with CCND1 amplification, 9 patients (50%) had CCND1 amplification in this study. However, of these 18 patients, all patients had ORAOV1 amplification, 13 patients (72%) had MYEOV amplification, 12 patients (67%) had FGFR1 amplification, and 10 patients (56%) had FGFR4 amplification. Moreover, of all patients, 7 patients (6%) had these five amplifications in FGF19, FGFR4, MYEOV, CCND1, and ORAOV1. The results suggested that the interactions of several genetic alterations located on 11q13 might be associated with a poor PFS and the development of ESCC. In addition, FGF19 and FGFR4 has been shown to stimulate epithelial-mesenchymal
transition during tumor progression\textsuperscript{20,21,43} and MYEOV, CTTN, and ORAOV1 has been reported to play critical oncogenic functions in invasion and metastasis.\textsuperscript{31,36,44} It suggested that these amplifications in \textit{FGF19}, \textit{FGF4}, \textit{MYEOV}, \textit{CTTN}, and \textit{ORAOV1} might be biomarkers at a premetastasis stage of cancer progression. We further showed the association between these genomic alterations and PFS in each treatment group. The results obtained from all populations in the surgery and CRT groups were similar with those from the patients in surgery group. The patients in the CRT group showed a trend for lower HRs compared with patients in the surgery group, except CTTN. However, the number of patients who received CRT is only 28 patients and it is difficult to conclude the biomarker specific within the patients with CRT. Therefore, we concluded that CNV amplification in \textit{FGF19}, \textit{FGF4}, \textit{MYEOV}, \textit{CTTN}, and \textit{ORAOV1} was a poor prognostic marker in T1bN0M0 ESCC patients.

High TMB leads to the creation of neoantigens and increases tumor immunogenicity; consequently, TMB has been reported as a biomarker for predicting the efficacy of ICIs in various tumors.\textsuperscript{45,46} In esophageal cancer patients treated with ICIs, TMB-high (≥10 mut/ Mb) patients were not significantly associated with a better PFS or OS than other patients.\textsuperscript{47} Cui et al revealed that TMB-high (≥10 mut/ Mb) patients had a significantly worse OS than other patients (HR, 1.87; 95% CI, 1.14-3.08, \(P = .011\)).\textsuperscript{48} In the present study, patients with a higher TMB (TMB ≥3/Mb) tended to have a better PFS than those with a lower TMB (TMB <3/Mb), but the difference was not statistically significant. Whether a TMB-high status might be a predictive marker for survival among patients with ESCC remains unclear. In addition, the optimal TMB cut-off needs to be explored in future studies.

Our study had several limitations. First, we could only obtain samples from 33.5% of the patients who were enrolled in the JCOG0502 trial, despite the high-quality clinical data obtained in this prospective study. Second, we might not have detected all the genetic alterations. The frequency of mutations in \textit{TP53}, which is essential in promoting the development of ESCC, has been reported to be 77%-97% in ESCC patients, including those with T1 ESCC.\textsuperscript{9,49,50} Our study showed that the rate of mutations in \textit{TP53} was only 35%. We analyzed archival FFPE tumor tissues, therefore the duration of preservation and method of fixation of our tissue samples might have influenced DNA quality. Finally, data on the expressions of proteins derived from the genomic alterations detected in this study were unknown. The present results should be validated in other cohorts and settings.

In conclusion, we found that genomic alterations in \textit{FGF19}, \textit{FGF4}, \textit{MYEOV}, \textit{CTTN}, and \textit{ORAOV1} were associated with poor PFS in patients with T1bN0M0 ESCC. These alterations could be novel targets for the development of new therapeutic agents for ESCC patients.
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DISCLOSURE

The authors have no conflicts of interest.

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**SUPPORTING INFORMATION**

Additional supporting information may be found in the online version of the article at the publisher’s website.

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