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Original Article

A 35-gene mutation profile predicts the therapeutic outcome of patients with esophageal squamous cell carcinoma receiving neo-adjuvant chemoradiation

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Abstract: Esophageal cancer is a common malignancy worldwide with a poor prognosis without radical resection. Neoadjuvant concurrent chemoradiotherapy (NACRT) followed by esophagectomy is widely used for treating locally advanced esophageal cancer in the thorax. The study aimed to assess mutation profiles and their correlation with therapeutic outcomes in patients diagnosed with locally advanced thoracic esophageal squamous cell carcinoma (ESCC). A retrospective analysis was conducted on 62 patients with ESCC who underwent NACRT. All patients received concurrent chemoradiotherapy (CCRT) utilizing intensity-modulated radiation therapy alongside concurrent chemotherapy with a cisplatin-based regimen. A 35-gene next-generation sequencing (NGS) panel detecting 402 genetic variants was used, which has been proven predictive in ESCC patients who received definitive chemoradiation. The 35-gene mutation profiles were analyzed in pre-treatment biopsies. The results reveled there were variants correlated with pathological complete remission or partial response, overall survival, and progression-free survival. A combination of p.Pro1319Ser and p.Arg2159Gly mutations in the *MUC17* gene demonstrated an adverse impact on pathological response (OR [95% CI] = 7.00 (3.07-15.94), $P < 0.001$). Additionally, the variants located in the *MUC17*, *MUC4*, and *MYH4* genes exhibited notably effects on tumor recurrence or mortality. Patients harboring either the MUC17 p.Thr2702Val or MUC4 p.Thr3355Ser mutation displayed a more than four-fold increased risk for disease recurrence or mortality. We concluded that specific mutations correlated to the pathological complete response in ESCC receiving neoadjuvant chemoradiation can be identified through the utilization of 35-gene expression profiles. Further investigation into the pathophysiological roles of *MUC17* and *MUC4* mutations in ESCC is warranted.

Keywords: Esophageal cancer, squamous cell carcinoma, neoadjuvant concurrent chemoradiotherapy (NACRT), next-generation sequencing (NGS)

Introduction

Esophageal cancer (EC) is a malignancy with a rising incidence and significant risk of recurrence and metastasis globally, particularly affecting the male population. It ranks seventh in terms of incidence and sixth in cancer-related mortality worldwide [1]. There are two primary histopathological types of primary EC: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma. ESCC constitutes 90% of EC cases and is predominant among non-Caucasian males, especially in Asian regions [1]. Diagnosis often occurs at an advanced stage (AJCC [American Joint Committee on Cancer] stage ≥ T2 or N+, M0) [2], due to the absence of early symptoms.

Trimodality therapy, including neoadjuvant concurrent chemoradiotherapy (CCRT) (NACRT) followed by esophagectomy, has shown benefits in downstaging and improving survival rates in locally advanced thoracic EC, as evidenced by randomized clinical trials and meta-analyses

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[3-5]. NACRT followed by esophagectomy has become a standard treatment approach for locally advanced thoracic EC [3-5]. The post-NACRT radical esophagectomy with complete resection is a major prognostic factor, indicating the importance of tumor removal with a free surgical margin [6, 7]. Pathological complete response (pCR) has been considered an significant prognostic factor for long-term and progression-free survival in patients with ESCC [8, 9]. However, only a minority (approximately 15-40%) of patients achieve pCR after preoperative chemoradiation, indicating a limited benefit from this treatment regimen [3, 10-12]. Conversely, most patients do not respond to preoperative chemoradiation, exposing them to potential morbidity, treatment-related toxicity, delayed surgery, and higher postoperative complication rates. Thus, the efficacy of preoperative chemoradiation appears unfavorable for non-responders.

Unlike esophageal adenocarcinoma, which benefits from anti-HER2 trastuzumab as a targeted therapeutic agent [13], ESCC lacks efficient targeted drugs despite increasing knowledge of its molecular alterations [14]. Currently, immunotherapy, either alone or combined with platinum and fluoropyrimidine-based chemotherapy, provides alternative options for ESCC patients, highlighting the growing importance of biomarker-based personalized treatment strategies.

While biomarkers predicting chemoradiotherapy response and prognosis in ESCC have been extensively studied [15, 16]. However, few studies have explored global genetic biomarkers for predicting CCRT response and clinical outcome using next-generation sequencing (NGS) techniques. NGS has become a powerful approach to diagnostics and to identify novel mutations. Meanwhile, there is no useful companion diagnostic panel to evaluate the treatment response and prognosis of ESCC patients. To make the NGS-based diagnostic tool possible for advanced ESCC, we initiated a study systematically exploring the potential biomarkers in the diagnosis of therapeutic responses and monitoring the recurrence of the disease by NGS using a custom ESCC panel. The panel comprising 402 genetic variants which covering 35 genes (listed in "materials and methods") that have been found frequently mutated in ESCC [17]. Combining radiotherapy dosimetry with the 35-gene mutation profile shows promise in developing a predictive model for therapeutic outcomes in patients with ESCC [18].

Materials and methods

Study population

A cohort of 62 patients diagnosed with locally advanced ESCC (T3N0-1M0 or T1-3N1M0) who underwent NACRT followed by esophagectomy were retrospectively examined at MacKay Memorial Hospital in Taiwan, with approval from the hospital's ethical committee. The prescribed radiation doses for gross tumors and metastatic lymph nodes or subclinical mucosal/submucosal disease and regional lymphatic basins were 48 and 43.2 Gy, respectively. All patients received CCRT using intensity-modulated radiation therapy in 24 fractions and concurrent chemotherapy with cisplatin-based regimen.

Among these patients, 22 (35.5%) exhibited a complete response to NACRT (defined as tumor regression grade-pCR), while the remaining enrolled patients (N = 40, 64.5%) showed a partial response (PR) to the treatment. Demographic and clinical data were obtained from medical records and the clinical database in the Department of Surgery and the Department of Pathology and Laboratory Medicine at MacKay Memorial Hospital. Formalin-fixed paraffin-embedded (FFPE) esophageal tumor tissue specimens before CCRT treatment were collected from endoscopic biopsies.

Genomic DNA extraction

For each specimen, 5 × 5 mm^2 sections with 5 μm thickness were cut from the FFPE block. Genomic DNA extraction was performed using the Cobas® DNA Sample Preparation Kit (Roche, Basel, Switzerland) following the manufacturer's instructions. The isolated DNA was quantified using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and DNA concentration and integrity were assessed using the DNF-474 High Sensitivity NGS Fragment Analysis Kit (AATI, Ankeny, IA, USA) and the Fragment Analyzer Automated CE System.

Table 1. Patient characteristics

Variables	N (%)	PR N = 40 (64.5)	pCR N = 22 (35.5)	*p-value**
Sex				0.124
Male	58 (93.5)	39 (97.5)	19 (86.4)	
Female	4 (6.5)	1 (2.5)	3 (13.6)	
cT				0.190
T1+T2	18 (29.0)	11 (27.5)	7 (31.8)	
T3	38 (61.3)	23 (57.5)	15 (68.2)	
T4	6 (9.7)	6 (15.0)	0 (0)	
cN				0.142
N0+N1	32 (51.6)	22 (55.0)	10 (45.5)	
N2	22 (35.5)	11 (27.5)	11 (50.0)	
N3	8 (12.9)	7 (17.5)	1 (4.5)	
cStage				0.561
cStage 2	18 (29.0)	13 (32.5)	5 (22.7)	
cStage 3	44 (71.0)	27 (67.5)	17 (77.3)	

*Pearson's Chi-square test or Fisher's exact test was employed for comparisons between the PR and CR groups. PR, partial response; CR, complete remission.

Table 2. Association between genetic variations and response to CCRT in patients with ESCC

Variants	PR 40 (64.5)	pCR 22 (35.5)	*p-value**
FAT p.Asn2678Asp	36 (72.0)	14 (28.0)	0.019
MUC17 p.Ala1322Thr	36 (70.6)	15 (29.4)	0.042
MUC17 p.Arg2159Gly	21 (84.0)	4 (16.0)	0.014
MUC17 p.Asn2706Ser	14 (87.5)	2 (12.5)	0.034
MUC17 p.Gly1307Ser	20 (80.0)	5 (20.0)	0.057
MUC17 p.Leu2703_Ieu	13 (86.7)	2 (13.3)	0.062
MUC17 p.Leu2712Val	14 (87.5)	2 (12.5)	0.034
MUC17 p.Pro1319Ser	28 (82.4)	6 (17.6)	0.001
MUC17 p.Pro1321Thr	26 (81.3)	6 (18.8)	0.004
MUC17 p.Pro2716Ala	13 (86.7)	2 (13.3)	0.062
MUC17 p.Ser2785Ala	16 (80.0)	4 (20.0)	0.095
MUC17 p.Thr2702Val	21 (80.8)	5 (19.2)	0.032
MUC17 p.Thr2721Ile	28 (75.7)	9 (24.3)	0.025
MUC17 p.Thr2802Ile	7 (100.0)	0 (0)	0.044
MUC17 p.Val1309Met	21 (80.8)	5 (19.2)	0.032
MUC4 p.Ala2409Val	1 (20.0)	4 (80.0)	0.049
MYH4 p.Gln1210AlafsTer3	7 (46.7)	8 (53.3)	0.097
PIK3CA p.Gly1049Cys	27 (73.0)	10 (27.0)	0.090
TTN p.Asp7145His	29 (72.5)	11 (27.5)	0.076

*Pearson's Chi-square test or Fisher's exact test.

Library construction and NGS

Genetic variant analysis by NGS was conducted at the laboratories of LIHPAO Life Science Co. The workflow included genomic DNA extraction, library preparation, sequencing, and data analysis. Each step followed previous reports [18] and briefly described as follows: 10 ng of DNA from an FFPE tissue sample was used to construct an amplicon library using the Ion AmpliSeqTM Library Kit 2.0 (Thermo Fisher Scientific) and Ion XpressTM Barcode Adapters Kit (Thermo Fisher Scientific). Library purification was performed using Agencourt AMPure XP reagent (Beckman Coulter, Brea, CA, USA) and washed with 70% ethanol on a DynaMagTM-2 Magnet (Thermo Fisher Scientific). Library quality control was conducted using the Ion Library TaqMan Quantitation Kit with the 7500 Fast Real-Time PCR System (Thermo Fisher Scientific).

The ESCC panel comprises 402 genetic variants, 159 amplicons, covering 35 genes, including *ABCA13*, *DNAH5*, *FBXW7*, *FAT1*, *FAT3*, *GPR98*, *EP300*, *DMD*, *KDM6A*, *CSMD3*, *CDKN2A*, *KMT2D*, *MUC4*, *MUC17*, *MUC2*, *MUC16*, *MYH4*, *TNN*, *HMCN1*, *USH2A*, *LRP1B*, *XIRP2*, *LRP2*, *NFE2L2*, *NOTCH*, *TTN*, *FSIP2*, *SI*, *PIK3CA*, *RB1*, *TP53*, *ZFHX4*, *TRIO*, *SYNE1*, and *PCLO*. The quantified libraries were clonally amplified on ion sphere particles by emulsion polymerase chain reaction using the Ion OneTouch™ 2 system with the Ion PGM Hi-Q View OT2 Kit (Thermo Fisher Scientific). Next, the ion sphere particles were enriched in an Ion OneTouch™ ES instrument (Thermo Fisher Scientific). Finally, the enriched ion sphere particles were loaded onto the 316 chip, and sequencing was performed on an Ion Torrent PGM system (Ion Torrent, Paisley, UK).

Table 3. Risk variants for non-complete (partial) response analyzed by univariate logistic regression

Variants	OR (95% CI)	*P-value*^
FAT1 p.Asn2678Asp	5.14 (1.33-19.83)	0.017*
MUC17 p.Ala1322Thr	4.2 (1.07-16.50)	0.040*
MUC17 p.Arg2159Gly	4.97 (1.42-17.34)	0.012*
MUC17 p.Asn2706Ser	5.39 (1.10-26.46)	0.038*
MUC17 p.Gly1307Ser	3.40 (1.05-11.00)	0.041*
MUC17 p.Leu2703_leu	4.82 (0.98-23.78)	0.054
MUC17 p.Leu2712Val	5.39 (1.10-26.46)	0.038*
MUC17 p.Pro1319Ser	6.22 (1.96-19.78)	0.002**
MUC17 p.Pro1321Thr	4.95 (1.58-15.50)	0.006**
MUC17 p.Pro2716Ala	4.82 (0.98-23.78)	0.054
MUC17 p.Ser2785Ala	3.00 (0.86-10.52)	0.086
MUC17 p.Thr2702Val	3.76 (1.16,12.16)	0.027*
MUC17 p.Thr2721Ile	3.37 (1.13-9.99)	0.028*
MUC17 p.Thr2802Ile	-	0.999
MUC17 p.Val1309Met	3.76 (1.16-12.16)	0.027*
MUC4 p.Ala2409Val	0.12 (0.01-1.11)	0.061
MYH4 p.Gln1210AlafsTer3	0.37 (0.11-1.22)	0.103
PIK3CA p.Gly1049Cys	2.49 (0.86-7.26)	0.094
TTN p.Asp7145His	2.64 (0.89-7.81)	0.080

^Univariate Cox regression. *$P < 0.05$; **$P < 0.01$. OR, odds ratio; CI, confidence interval.

Table 4. Collinearity diagnostics of MUC17 variation

Variants	VIF
MUC17 p.Arg2159Gly	15.852
MUC17 p.Gly1307Ser	8.131
MUC17 p.Val1309Met	24.117

VIF, variance inflation factor.

The DNA sequencing data generated using the personal genome machine were analyzed using Torrent Suite software (Thermo Fisher Scientific). Variant calling and annotation were performed using Ion-Reporter v5.1.0. Mutations with an average coverage of ≥ 1500 reads and a mutant allele frequency of ≥ 5% were reported.

Statistical analysis

The distribution of demographic and clinical characteristics, along with variant genotypes among subgroups with different clinical outcomes, such as treatment response, recurrence, and mortality, was analyzed using Pearson's Chi-square test or Fisher's exact test if any cell counts in the cross table were less than 5. Univariate or multivariate logistic regression was employed to evaluate the odds ratios [19] of partially responding (PR) to CCRT. Hazard ratios (HRs) obtained from Cox regression analysis were utilized to depict the relative risk of recurrence or death. Data were expressed as mean values and 95% confidence intervals (CIs) for regression analysis. Correlations between variant genotypes and both overall survival (OS) and progression-free survival (PFS) were analyzed using the Kaplan-Meier survival function and compared using the log-rank test. The linear relationship between genotypic variables was assessed by collinearity diagnostics using the variance inflation factor (VIF).

Receiver operating characteristic (ROC) curve analysis was employed to evaluate the predictive performance of risk genotypes for PR, recurrence, and mortality. The area under the curve (AUC) of the ROC curve was used to assess the discriminatory capability of the related genotypes for patients with ESCC. An AUC of 0.7-0.8 is generally considered acceptable, while 0.8-0.9 is deemed excellent [20].

All statistical analyses were performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA). A p-value ≤ 0.05 was considered statistically significant.

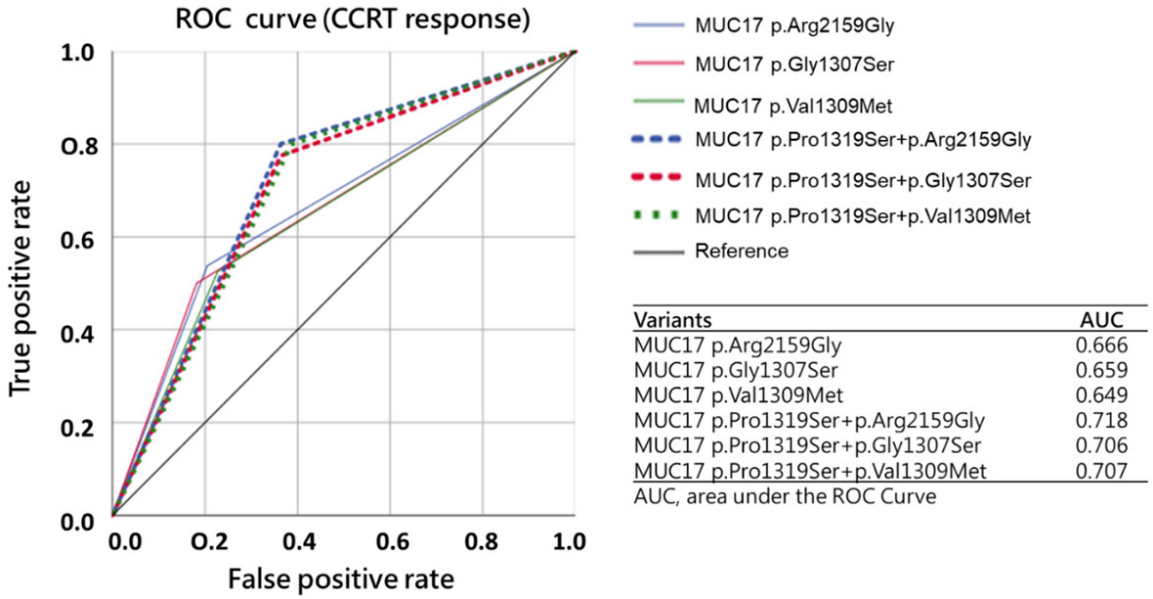
Results

The characteristics of the patients are outlined in **Table 1**. Among the 62 patients, 58 (93.5%) were male, with 38 (61.3%) presenting a T3 clinical tumor stage (cT), while 32 patients exhibited N0 or N1 clinical lymph node stage (cN). The resulting clinical stage (cStage) comprised 29% for stage 2 and 71.0% for stage 3, respectively. There was no notable difference in the distribution of these clinical or demographic variables between the groups showing PR or pCR (**Table 1**). **Table 2** illustrates 19 variants detected from tumor tissue before CCRT

Table 5. Distributions and risks for partial response to CCRT in patients with indicated genetic variations

Variants	Unfavorable	PR 40 (64.5)	pCR 22 (35.5)	*p-value**	OR (95% CI)	*p-value^*
MUC17 p.Pro1319Ser+p.Arg2159Gly	Negative	8 (20.0)	15 (68.2)		1	
	Positive	32 (80.0)	7 (31.8)	< 0.001	7.00 (3.07-15.94)	< 0.001
MUC17 p.Pro1319Ser+p.Gly1307Ser	Negative	9 (22.5)	14 (63.6)		1	
	Positive	31 (77.5)	8 (36.4)	0.002	6.03 (2.69-13.52)	< 0.001
MUC17 p.Pro1319Ser+p.Val1309Met	Negative	8 (20.0)	14 (63.6)		1	
	Positive	32 (80.0)	8 (36.4)	0.001	6.35 (2.81-14.39)	< 0.001

*Pearson's Chi-square test or Fisher's exact test. ^Univariate Cox regression. OR, odds ratio; CI, confidence interval.



Variants	AUC
MUC17 p.Arg2159Gly	0.666
MUC17 p.Gly1307Ser	0.659
MUC17 p.Val1309Met	0.649
MUC17 p.Pro1319Ser+p.Arg2159Gly	0.718
MUC17 p.Pro1319Ser+p.Gly1307Ser	0.706
MUC17 p.Pro1319Ser+p.Val1309Met	0.707

AUC, area under the ROC Curve

Figure 1. Receiver operating characteristic (ROC) curve illustrating the combined risk variations used to differentiate patients with ESCC with complete or partial response to CCRT. AUC, area under the ROC curve.

that exhibited significance ($P \leq 0.05$) associated with CCRT response, as determined by Pearson's Chi-square test or Fisher's exact test. Among these genetic variants, 14 were located within the coding region of the MUC17 gene. Univariate logistic regression further revealed 11 variants, including FAT1 p.Asn2678Asp, MUC17 p.Ala1322Thr, MUC17 p.Arg2159Gly, MUC17 p.Asn2706Ser, MUC17 p.Gly1307Ser, MUC17 p.Leu2712Val, MUC17 p.Pro1319Ser, MUC17 p.Pro1321Thr, MUC17 p.Thr2702Val, MUC17 p.Thr2721Ile, and MUC17 p.Val1309Met, significantly correlated with an increased risk of PR. Of these variants, MUC17 p.Pro1319Ser exhibited the most notable correlation with a 6.22-fold increased risk for PR (OR [95% CI] = 6.22 (1.96-19.78), P = 0.002, **Table 3**).

Multicollinearity refers to a high degree of linear intercorrelation and can lead to inaccurate results in multivariable regression analyses [21]. We assessed the collinearity of unfavorable variants for CCRT response within the MUC17 gene using collinearity diagnostics, examining the VIF. Three variants with a predominant unfavorable effect, including MUC17 p.Arg2159Gly, p.Gly1307Ser, and p.Val1309Met, exhibited a high degree of genetic collinearity (**Table 4**). Combining each of these three variants with p.Pro1319, patients carrying at least one of the variations (positive) showed a significantly increased PR group compared to those carrying none of the variations (negative), especially the combination of MUC17 p.Pro1319Ser and p.Arg2159Gly (PR vs. pCR, 80% vs. 31.8%, P < 0.001, **Table 5**). Univariate

Table 6. Correlation between genetic variants and disease recurrence in patients with ESCC

Genetic variants	No recurrence 11 (17.7)	Recurrence 51 (82.3)	*p*-value*
EP300 p.Glu1523Lys	5 (50.0)	5 (50.0)	0.011
MIR548N|TTN-AS1|TTN|p.Thr19762Ile	9 (15.3)	50 (84.7)	0.079
MIR548N|TTN-AS1|TTN|p.Thr21880Ile	9 (15.3)	50 (84.7)	0.079
MUC16 p.Thr5382Lys	4 (57.1)	3 (42.9)	0.015
MUC17 p.Asn2706Ser	0 (0)	16 (100.0)	0.052
MUC17 p.Gly2906Ala	2 (66.7)	1 (33.3)	0.079
MUC17 p.Leu2712Val	0 (0)	16 (100.0)	0.052
MUC17 p.Lys1306Asn	3 (60.0)	2 (40.0)	0.035
MUC17 p.Thr2702Val	0 (0)	26 (100.0)	0.002
MUC17 p.Thr2721Ile	3 (8.1)	34 (91.9)	0.021
MUC17 p.Asn2706Ser	0 (0)	16 (100.0)	0.052
MUC17 p.Leu2703_Leu2704delinsProVa	0 (0)	15 (100.0)	0.052
MUC17 p.Pro2716Ala	0 (0)	15 (100.0)	0.052
MUC17 p.Thr2721Ile	3 (8.1)	34 (91.9)	0.021
MUC4 p.Ala2390Thr	4 (57.1)	3 (42.9)	0.015
MUC4 p.Asp2397His	5 (62.5)	3 (37.5)	0.003
MUC4 p.Gly3372Asp	4 (40.0)	6 (60.0)	0.067
MUC4 p.His2381Asp	5 (62.5)	3 (37.5)	0.003
MUC4 p.His2413Gln	3 (60.0)	2 (40.0)	0.035
MUC4 p.Pro3360His	7 (36.8)	12 (63.2)	0.026
MUC4 p.Ser3370Thr	3 (50.0)	3 (50.0)	0.063
MUC4 p.Thr2382Ala	6 (46.2)	7 (53.8)	0.007
MUC4 p.Thr2398Ala	3 (100.0)	0 (0)	0.004
MUC4 p.Thr2411Ser	9 (39.1)	14 (60.9)	0.001
MUC4 p.Thr3350Asn	3 (50.0)	3 (50.0)	0.063
MUC4 p.Thr3355Ser	11 (35.5)	20 (64.5)	< 0.001
MUC4 p.Val3353Ala	6 (40.0)	9 (60.0)	0.018

*Pearson's Chi-square test or Fisher's exact test.

logistic regression also demonstrated the pronounced effect of MUC17 p.Pro1319Ser and p.Arg2159Gly, with a seven-fold increased risk for PR (OR [95% CI] = 7.00 (3.07-15.94), $P < 0.001$, **Table 5**). ROC curve analysis revealed that positivity for either MUC17 p.Pro1319Ser or p.Arg2159Gly had a fair capability for predicting CCRT response (AUC = 0.718, **Figure 1**).

Furthermore, we analyzed the correlation between genetic variation and prognosis, including tumor recurrence and mortality. **Table 6** indicates that 24 and 16 variants detected in tumor tissue before CCRT showed a significant correlation with tumor recurrence. Most variants were within the coding regions of the MUC17 and MUC4 genes. Univariate logistic regression further revealed that 15 variants of the tissue before CCRT, including MUC17 p.Asn2706Ser, MUC17 p.Leu2712Val, MUC17 p.Thr2702Val, MUC17 p.Thr2721Ile, MUC17 p.Asn2706Ser, MUC17 p.Pro2716Ala, MUC17 p.Thr2721Ile, MUC4 p.Asp2397His, MUC4 p.His2381Asp, MUC4 p.Pro3360His, MUC4 p.Thr2382Ala, MUC4 p.Thr2411Ser, MUC4 p.Thr3355Ser, and MUC4 p.Val3353Ala, were significantly correlated with an increased risk for disease recurrence (**Table 7**).

Similar findings were observed in the analysis of patient survival by univariate logistic regression. A total of 13 variants were significantly associated with an increased risk of patient mortality, as detected from the tissue before CCRT. Most variants were located within the coding regions of the MUC17 and MUC4 genes. MUC17 p.Thr2702Val, detected from tissues, emerged as the predominant variant for patient

Table 7. Risk genetic variants of tumor tissues before CCRT for disease recurrence analyzed by univariate Cox regression

Variants	HR (95% CI)	*P-value**
EP300 p.Glu1523Lys	0.41 (0.16-1.03)	0.058
MIR548N|TTN-AS1|TTN|p.Thr19762Ile	5.09 (0.70-37.10)	0.108
MUC16 p.Thr5382Lys	0.32 (0.10-1.01)	0.051
MUC17 p.Asn2706Ser	3.85 (2.03-7.32)	< 0.001
MUC17 p.Gly2906Ala	0.22 (0.03-1.57)	0.129
MUC17 p.Leu2712Val	3.52 (1.86-6.69)	< 0.001
MUC17 p.Lys1306Asn	0.35 (0.09-1.45)	0.149
MUC17 p.Thr2702Val	3.32 (1.84-6.00)	< 0.001
MUC17 p.Thr2721Ile	2.28 (1.27-4.11)	0.006
MUC17 p.Asn2706Ser	3.85 (2.03-7.32)	< 0.001
MUC17 p.Leu2703_Leu2704delinsProVa	3.61 (1.89-6.90)	< 0.001
MUC17 p.Pro2716Ala	3.61 (1.89-6.90)	< 0.001
MUC17 p.Thr2721Ile	2.28 (1.27-4.11)	0.005
MUC4 p.Ala2390Thr	0.34 (0.11-1.10)	0.072
MUC4 p.Asp2397His	0.28 (0.09-0.91)	0.034
MUC4 p.Asp2405Asn	0.67 (0.16-2.76)	0.577
MUC4 p.Gly3372Asp	0.50 (0.21-1.16)	0.107
MUC4 p.His2381Asp	0.28 (0.09-0.89)	0.031
MUC4 p.His2413Gln	0.32 (0.08-1.31)	0.113
MUC4 p.Pro3360His	0.46 (0.24-0.88)	0.020
MUC4 p.Ser3370Thr	0.39 (0.12-1.24)	0.110
MUC4 p.Thr2382Ala	0.35 (0.16-0.79)	0.011
MUC4 p.Thr2398Ala	0.04 (0.00-2.76)	0.138
MUC4 p.Thr2411Ser	0.46 (0.25-0.86)	0.015
MUC4 p.Thr3350Asn	0.39 (0.12-1.24)	0.110
MUC4 p.Thr3355Ser	0.39 (0.21-0.69)	0.002
MUC4 p.Val3353Ala	0.48 (0.23-1.00)	0.049
USH2A|LOC102723833 p.Leu1658Pro|	0.58 (0.33-1.01)	0.054

*Univariate Cox regression. HR, hazard ratio; CI, confidence interval.

survival (OR [95% CI] = 4.22 (2.20-8.11), P < 0.001, **Table 8**). Additionally, MUC4 p.Thr3355Ser exhibited a reduced risk for death (OR [95% CI] = 0.40 (0.21-0.74), P = 0.004, **Table 8**). Notably, the variant MYH4 p.Gln1210AlafsTer3 within the MYH4 gene also showed a significant correlation with a reduced risk of patient mortality (OR [95% CI] = 0.38 (0.17-0.87), P = 0.022, **Table 8**).

We assessed the collinearity of unfavorable variants for prognosis response within both the MUC17 and MUC4 genes using collinearity diagnostics. None of the variants displayed a high degree of genetic collinearity (data not shown). We designated the variant of MUC17 p.Thr2702Val and the wild type of MUC4 p.Thr3355Ser as unfavorable genotypes. Patients carrying at least one of the unfavorable genotypes (positive) demonstrated 100% sensitivity in predicting disease recurrence in tumor tissue before CCRT (P < 0.001, **Table 9**, Fisher's exact test). Univariate Cox regression further revealed a 4.57-fold increased hazard for recurrence detected from fresh esophageal tumor tissue (HR [95% CI] = 4.57 (2.31-9.01), P < 0.001, **Table 10**).

MUC17 p.Thr2702Val and MUC4 p.Thr3355Ser also predicted the risk of death with 92.1% sensitivity in tumor tissues before CCRT treatment (P < 0.001, **Table 9**). Univariate Cox regression also demonstrated a markedly increased risk of up to 4.98 folds for death in fresh tissues (HR [95% CI] = 4.98 (2.34-10.58), P < 0.001, **Table 10**).

ROC curve analysis revealed that positivity for either the unfavorable genotype of MUC17 p.Thr2702Val or MUC4 p.Thr3355Ser had excellent capability for predicting tumor recurrence (AUC = 0.873, **Figure 2A**) and fair discriminative ability for patient mortality (AUC = 0.787, **Figure 2B**). Patients with or without favorable genotypes exhibited a significant difference in the distribution of both PFS and OS (P < 0.001 by log-rank test, respectively). The median PFS was 35.7 and 10.2 months for the negative and positive groups, respectively (**Figure 3A**). Meanwhile, none of the unfavorable genotype carriers reached 5-year PFS, in contrast to negative patients, who had a 41.0% 5-year PFS rate (**Figure 3A**). For OS analysis, patients without any of the unfavorable genotypes enjoyed a median OS of 68.4 months and a 57.2% long-term survival. Conversely, those carrying an unfavorable genotype only had an 18-month OS and 3.1% long-term survival (**Figure 3B**).

Table 8. Risk genetic variants of the tumor tissues before CCRT for mortality by univariate Cox regression

Variants	HR (95% CI)	*P-value**
EP300 p.Glu1523Lys	0.61 (0.24-1.54)	0.263
MUC16 p.Thr5382Lys	0.46 (0.14-1.49)	0.195
MUC17 p.Asn2706Ser	4.13 (2.14-7.97)	< 0.001
MUC17 p.Leu2703_Leu2704delinsProVal	3.86 (1.99-7.47)	< 0.001
MUC17 p.Leu2712Val	4.00 (2.07-7.74)	< 0.001
MUC17 p.Pro1319Ser	2.13 (1.16-3.92)	0.015
MUC17 p.Pro2716Ala	3.86 (1.99-7.47)	< 0.001
MUC17 p.Ser656Thr	0.05 (0.00-370.06)	0.505
MUC17 p.Thr2702Val	4.22 (2.20-8.11)	< 0.001
MUC17 p.Thr2721Ile	3.32 (1.68-6.57)	0.001
MUC4 p.Ala2390Thr	0.33 (0.08-1.38)	0.130
MUC4 p.Asp2397His	0.29 (0.07-1.18)	0.084
MUC4 p.Gly3372Asp	0.60 (0.24-1.52)	0.280
MUC4 p.His2381Asp	0.28 (0.07-1.15)	0.077
MUC4 p.His2413Gln	0.23 (0.03-1.67)	0.146
MUC4 p.Pro3360His	0.47 (0.23-0.96)	0.037
MUC4 p.Ser3370Thr	0.53 (0.17-1.73)	0.295
MUC4 p.Thr2382Ala	0.35 (0.14-0.89)	0.028
MUC4 p.Thr2398Ala	0.04 (0-6.11)	0.214
MUC4 p.Thr2411Ser	0.50 (0.26-0.98)	0.042
MUC4 p.Thr3350Asn	0.53 (0.17-1.73)	0.295
MUC4 p.Thr3355Ser	0.40 (0.21-0.74)	0.004
MUC4 p.Val3353Ala	0.34 (0.14-0.80)	0.014
MYH4 p.Gln1210AlafsTer3	0.38 (0.17-0.87)	0.022
PCLO p.Val196Phe	0.04 (0.00-8.88)	0.248
USH2A|LOC102723833 p.Leu1658Pro|	0.57 (0.31-1.03)	0.060

*Univariate Cox regression. HR, hazard ratio; CI, confidence interval.

Discussion

The prognosis of advanced ESCC remains poor despite advancements in multimodality therapy, with an average 5-year survival rate of around 20% [22, 23]. However, immune checkpoint inhibitors such as pembrolizumab and nivolumab have shown promising response rates in advanced EC [24-26], leading to their approval by the FDA and in many countries for ESCC treatment. Consequently, the need for useful biomarkers to monitor treatment response and clinical outcomes has become increasingly critical for personalized therapeutic strategies in this complex disease.

In this study, we demonstrated novel genetic variants correlated with treatment response and prognosis in patients with ESCC undergoing CCRT followed by esophagectomy, utilizing the 35-gene expression profile. We identified 11 variants, primarily within the coding region of the MUC17 gene that significantly correlated with an increased risk of PR, with MUC17 p.Pro1319Ser exhibiting the most predominant effect. Furthermore, combined analysis of MUC17 p.Pro1319Ser and p.Arg2159Gly showed a more pronounced effect. Additionally, variants located in the coding regions of MUC17, MUC4, USH2A, and MYH4 exhibited significant effects on tumor recurrence or mortality, with a combined analysis of MUC17 p.Thr2702Val and MUC4 p.Thr3355Ser in fresh tissue revealing a predominant effect on recurrence and death.

Among these variants, MUC17 p.Arg2159Gly, p.Gly1307Ser, and p.Val1309Met showed a strong positive linear correlation. Variant p.Arg2159Gly (A to G, rs28555173) could be a naturally occurring variant, a single-nucleotide polymorphism (SNP; rs28555173) with a minor allele frequency of around 0.2 in East Asians. Both p.Gly1307Ser and p.Val1309Met represent transition mutations from G to A (C to T) [27]. The strong intercorrelation among these three variants, characterized by purine-to-purine transition, suggests a shared mechanism influencing the somatic mutation profile. The expression or activity of Zn2+-dependent DNA cytosine deaminases (encoded by the APOBEC protein family) as well as thymine-DNA glycosylase have been associated with such transition mutations [28, 29].

MUC17 and MUC4 encode mucin 17 and mucin 4, respectively. Mucins are proteins that cover and protect epithelial cells and are closely involved in inflammation and cancer [30]. Certain mucins are considered drug targets, and inhibiting mucin function has shown the ability to block tumorigenicity in experimental models [30]. Mucin 4 has been found to pro-

Table 9. Distribution of patients with or without unfavorable genotypes in tissue before CCRT by disease recurrence and mortality

Variants^	Unfavorable genotype	No recurrence 11 (17.7)	Recurrence 51 (82.3)	*p-value**	Alive 11 (17.7)	Dead 51 (82.3)	*p-value**
MUC17 p.Thr2702Val (v)+	Negative	11 (45.8)	13 (54.2)	< 0.001	13 (54.2)	11 (45.8)	< 0.001
MUC4 p.Thr3355Ser (w)	Positive	0 (0)	38 (100)		3 (7.9)	35 (92.1)	

*Pearson's Chi-square test or Fisher's exact test. ^Unfavorable genotypes: MUC17 p.Thr2702Val (v, variation) and MUC4 p.Thr3355Ser (w, wild-type).

Table 10. Unfavorable genotype of tissue before CCRT for the risk of recurrence and death, analyzed by univariate Cox regression

Variants	Unfavorable genotype	HR (95% CI)# Recurrence	*P-value**	HR (95% CI)^ Dead	*P-value**
MUC17 p.Thr2702Val (v)+	Negative	1		1	
MUC4 p.Thr3355Ser (w)	Positive	4.57 (2.31-9.01)	< 0.001	4.98 (2.34-10.58)	< 0.001

*Univariate Cox regression. #Hazard ratio (HR [95% confidence interval (CI)]) of disease recurrence. ^Hazard ratio (HR [95% confidence interval (CI)]) of mortality. Unfavorable genotypes: MUC17 p.Thr2702Val (v, variant) and MUC4 p.Thr3355Ser (w, wild-type).

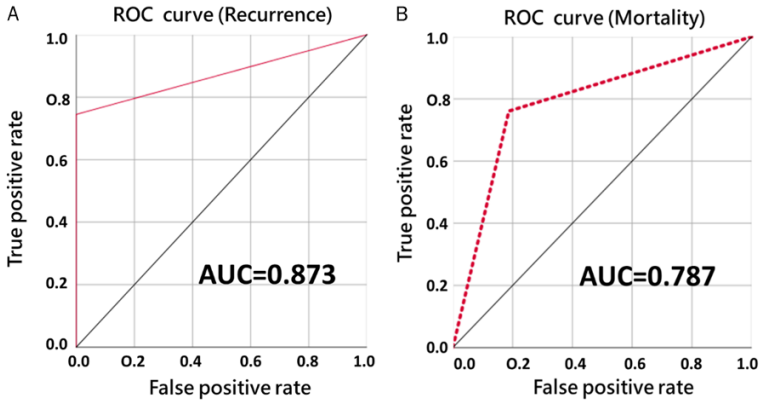


Figure 2. Receiver operating characteristic (ROC) curves depicting the risk genotypes of tissues before CCRT, used to differentiate patients with ESCC with (A) disease recurrence or non-recurrence and (B) dead or alive. AUC, area under the ROC curve.

mote tumorigenicity in epithelial carcinomas and cancer metastasis and is thus considered a valuable prognostic biomarker for cancer [19, 31, 32]. Mucin 17 has been reported to inhibit the progression of gastric cancer by limiting the inflammatory response [33]. A study analyzing the somatic mutation profile in Chinese patients with hepatocellular carcinoma by NGS also revealed the mutation profile of mucin genes, including MUC4, MUC17, MUC12, and MUC16. MUC17 was further found to have more co-mutations with TP53 [34]. A previous study using the same gene panel showed significantly higher mutation rates in MUC17 in the ESCC cohort compared to data from The Cancer Genome Atlas (79.5% vs. 5.7%) [18]. This study also demonstrated mutations in FSIP2 and SYNE1 as potential prognostic markers in patients with ESCC receiving definitive CCRT. However, similar results were not observed in our cohort receiving NACRT.

The mucins, encoded by different genes, share some structural similarities. Both MUC17 and MUC4 are type I membrane-anchored proteins with an N-terminal extracellular region and a C-terminal cytoplasmic tail, which can be released by cleavage [35]. The central tandem-repeat (TR) domains, rich in proline, threonine, and serine, known as the PTS domain, are characteristic features of all mucins [35, 36]. Threonine and serine residues in the TR region are potential sites for post-translational modification (PTM), such as O-glycosylation and phosphorylation, playing a crucial role in structural changes and partner interactions. Proline residues may also influence the proper packing of carbohydrate structures. Polymorphisms of VNTR (variable number of TRs) in mucin have been shown to contribute to the expression of polypeptides with different lengths [37].

Glycosylation, a major PTM, stabilizes proteins and makes them resistant to degradation.

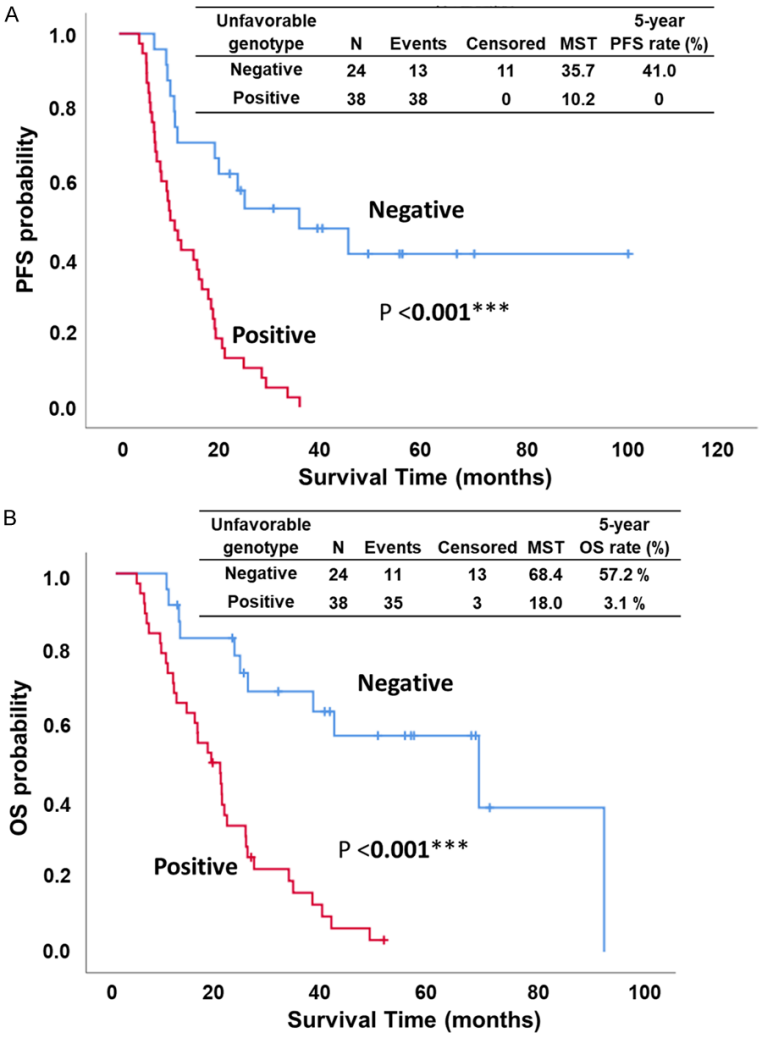


Figure 3. Kaplan-Meier estimates demonstrating tumor progression-free survival (PFS, A) and overall survival (OS, B) based on patients carrying unfavorable genotypes (positive) or not (negative) in tissues before CCRT. Unfavorable genotypes include MUC17 p.Thr2702Val (variant) and MUC4 p.Thr3355Ser (wild type). MST, median survival time.

O-glycosylation involves attaching an α-D-GalNAc (α-D-Nacetylgalactosamine) monosaccharide to the hydroxyl group of serine or threonine residues [24]. Threonine glycosylation induces different structural alterations than serine glycosylation, influencing water surrounding capabilities and lectin affinity [38, 39]. Proline residues around the glycosylation site are also crucial for O-glycosylation [40]. Phosphorylation mainly occurs at serine and threonine residues, particularly at serine residues. Phospho-tyrosine is less abundant compared to phospho-serine and phospho-threonine [41].

In our findings, the variation from proline to serine at residue P1319 (p.Pro1319Ser) in mucin 17 showed a significant correlation with a poor response to CCRT. This alteration might create a potential O-glycosylation site and a phosphorylation site, inducing conformational changes affecting the stability or expression of mucin 17 [42-44]. Epigenetic downregulation of MUC17 has been linked to acquired drug resistance to EGFR-TKI (gefitinib/osimertinib) in NSCLC [45]. Genetic variation may confer resistance of ESCC cells to CCRT via downregulation of mucin 17 expression. Whether increased mucin 17 expression enhances the treatment efficacy of CCRT in ESCC is worthy of further investigation.

Prognostic analysis revealed that a threonine-to-serine substitution at residue T3355 of the mucin 4 isoform precursor (NP_060876, MUC4 p.Thr3355Ser) plays a favorable role in the clinical outcome of patients with ESCC. Phosphorylation or glycosylation of serine instead of threonine may induce structural changes and modulate interacting partners and functions. Serine phosphorylation has been found to induce smaller rheostat-like changes, while phosphorylation at threonine is likely to exhibit a strong disorder-to-order transition [42].

Meanwhile, frame shift mutations in *MYH4* gene (p.Gln1210AlafsTer3, MYH4 c.3626dup) revealed an obvious correlation with reduced mortality risk. *MYH4* encodes the myosin heavy chain 4 protein, also known as myosin heavy chain 2b (MyHC-2b). Myosin heavy chain is the motor protein of muscle thick filaments and is involved in muscle contraction [46]. MYH4 mRNA expression tends to increase in ovarian

cancer compared to normal tissue [47]. Notably, MYH4 has been suggested as a potential driver gene involved in establishing the tumor microenvironment [48]. The frame shift mutation causing premature termination at residue 1,212 of MyHC-2b might increase tumor cell death, displaying a trend of good response to CCRT (**Table 3**), and favorable effects on the survival of patients (**Table 8**).

A growing body of research has identified predictive biomarkers for CCRT response and clinical outcomes in EC, many of which are germline SNPs or gene expression products related to DNA repair, receptor tyrosine kinase, and cytokine signaling [15, 16, 49]. Our study, however, identified novel somatic genetic variants in mucin genes, offering insights into predicting both treatment response and prognosis. These findings present new opportunities for advancing personalized treatment in patients with ESCC.

To the best of our knowledge, our study for the first time demonstrated that these genetic variants of fresh ESCC tissue within the coding region of *MUC17*, *MUC4*, and *MYH4* can predict CCRT response and prognosis in ESCC patients. We believe that a detection panel incorporating these genetic variations holds promise for precision medicine in ESCC. Immunotherapeutic agents may be considered as adjuncts to treatment regimens for patients with unfavorable genotypes in CCRT response. For those carrying poor prognostic genotypes, more careful and closer follow-up to monitor tumor recurrence is warranted.

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Disclosure of conflict of interest

None.

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