OncoTargets and Therapy

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ORIGINAL RESEARCH

Prognostic Implications of Six Altered Genes in Asian Non-Surgical Esophageal Carcinoma Patients Treated with Chemoradiotherapy

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Background: Esophageal cancer (EC), especially esophageal squamous cell carcinoma, remained as one of the most aggressive tumors in China with a five-year survival rate of around 40%. Molecular characteristics through next-generation sequencing are becoming an emerging method in identifying prognostic biomarkers for better treatment management for EC patients.

Methods: Targeted next-generation sequencing using a 422-gene pan-cancer panel was performed with tumor tissue samples from a total of 69 Asian non-surgical esophageal carcinoma patients (AEC) treated with chemoradiotherapy. A TCGA cohort of 143 EC patients and another Asian ESCC cohort of 47 patients were employed for validation.

Results: In the AEC cohort, alterations in *TP53* (94.2%) and *NOTCH1* (55.1%) were the two most frequently observed alterations, whereas in the TCGA cohort, only *TP53* alterations were observed at a high ratio (85.3%). Co-amplifications of *FGF19* and *CCND1* were found at a similar ratio in both cohorts. Multiple alterations in the DNA damage pathway were identified but not associated with overall survival in AEC. Using univariate and multivariate Cox regression analyses, six gene alterations including *YAP1* amplification, *RB1* alteration, *BAP1* mutation, *MYC* amplification, *WRN* mutation, and *BRIP1* mutation were identified as adverse prognostic factors in the AEC cohort. A Cox proportional hazard model based on the six prognosis-related genes was constructed and showed the ability in distinguishing EC patients with poorer disease outcomes in AEC and two validation cohorts.

Conclusion: Six gene alterations were found to be potential unfavorable prognostic markers that might provide guidance in the treatment management for EC patients.

Keywords: esophageal cancer, *YAP1*, *RB1*, *BAP1*, *MYC*, *BRIP1*, *WRN*, overall survival, chemoradiotherapy

Introduction

Esophageal cancer (EC) is the one of most aggressive tumors in China, with a higher ratio of age-standardized mortality rate in Chinese males compared to Chinese females as well as the UK, the USA and even worldwide.[1] Esophageal squamous cell carcinoma (ESCC) is more often observed in the Asian population, while esophageal adenocarcinoma (EAC), on the other hand, occurs mainly in North America and Western Europe.[1,2] Smoking and alcohol consumption are two high-risk factors for ESCC,[3] whereas obesity is a strong risk factor for EAC.[4] The 5-year survival rates of EC depend on several factors, including the stage of cancer at the time of diagnosis. In China, EC patients achieved a 5-year

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Received: 24 August 2021
Accepted: 26 December 2021
Published: 13 January 2022

survival rate of around 40%.[5,6] In the US, the 5-year survival rate is 47% for EC patients with only localized tumors and 20% for all EC patients combined according to SEER database (cancer.org).

According to the National Comprehensive Cancer Network (NCCN) guideline, surgery is a major treatment used for locally advanced resectable EC patients with additional preoperative chemoradiation or perioperative chemotherapy to improve survival.[7] Targeted treatment strategies have also been explored in EC patients including HER2-targeted therapy, anti-angiogenesis therapy, and immunotherapy. HER2 TKI trastuzumab has been approved by the FDA for *HER2*-positive advanced ECs.[8] Ramucirumab, a VEGFR-2 antibody, has been approved for pre-treated patients with advanced or metastatic EAC initially as monotherapy and subsequently as combination therapy with paclitaxel.[9] Pembrolizumab is approved in 2017 by the FDA for EC patients with high microsatellite instability and/or PD-L1 expression.[10]

With the aid of next-generation sequencing, molecular characteristics are becoming an emerging aspect in multidisciplinary treatment decision-making to create an overall therapeutic plan for cancer patients. The genomic landscape of EC genomes has been studied in both Asian and Western populations.[11,12] Highly mutated genes in EC included *TP53, NOTCH1, PIK3CA, RB1, CDKN2A* have been identified in different ethnic groups. The association between gene alterations and prognosis has been indicated.[13,14] Here, we performed targeted panel sequencing of tumor tissues from 69 Asian EC patients. A TCGA cohort of 143 EC patients was also included in this study for comparison. Six gene alterations were shown to be potential prognostic biomarkers for overall survival in EC patients using univariate and multivariate Cox regression analyses. The Cox proportional hazard model built on the six potential prognostic biomarkers demonstrated the ability to select patients with worse disease outcome in the EC patients.

Method

Patient Cohort

A total of 69 patients clinically diagnosed as non-surgical EC was retrospectively recruited from Shandong Provincial Hospital Affiliated to Shandong University according to the NCCN guidelines.[7] The inclusion criteria were as follows: 1) all patients had histologically proven primary non-surgical EC and 2) the patients were treated with chemoradiotherapy. The study was approved by the Ethical Review Board of Shandong Provincial Hospital, and informed written consent was obtained from each participant.

DNA Extraction and Library Preparation

As previously described,[15] the genomic DNA was extracted from formalin-fixed and paraffin-embedded (FFPE) using the QIAamp DNA FFPE Tissue Kit (Qiagen). The extracted DNA quantity was evaluated using a Qubit 3.0 fluorometer and extracted DNA quality was measured using Nanodrop 2000 (Thermo Fisher Scientific). Sequencing libraries were prepared using the KAPA Hyper Prep Kit (KAPA Biosystems) and sequenced with a pan-cancer panel of 422 genes.

Sequencing Data Analysis

Sequencing data analysis was performed as previously described.[15] In brief, FASTQ file quality control was performed with trimmomatic[16] (below 15 or N bases were removed). Reads were mapped to the reference Human Genome (hg19) using Burrows-Wheeler Aligner (BWA-mem, v0.7.12; https://github.com/lh3/bwa/tree/master/bwakit). VarScan2[17] was used for somatic mutation detection. Genome Analysis Toolkit was applied to local realignment around the indels and base quality score recalibration (GATK 3.4.0; https://software.broadinstitute.org/gatk/), which was also used for detecting germline mutations. Somatic variants were called if mutant allele frequency (MAF) was at least 0.2%, and at least three supporting-reads from both directions. Common SNPs were filtered out according to dbSNP (v137) and the 1000 Genomes database, and annotated using ANNOVAR.[18] Genomic fusions were identified by FACTERA[19] with default parameters. Copy-number variations (CNVs) were detected using ADTEx (http://adtex.sourceforge.net) with default parameters. Somatic CNVs were identified with the cut-off of 0.65 for copy-number loss and 1.50 for copy-number gain using paired normal/tumor samples for each exon.

Data Analysis

The Kaplan–Meier survival curves were performed to estimate OS in different genomic groups. The log-rank test was performed to analyze differences between groups. The univariate and multivariate analyses were performed to evaluate the prognostic value of clinicopathological characteristics and gene alterations on OS. Overall

survival (OS) was measured from the date of pathological diagnosis of EC to the date of death or last follow-up.

Backward stepwise selection with the Akaike information criterion (AIC) was used to identify variables for the multivariate Cox proportional hazards model. Model performance was evaluated by assessing discrimination against the index of concordance (C-index) and plotting Kaplan–Meier curves over the quartiles of prediction by nomogram.[20] The optimal cutoff for risk score was selected using X-tile based on the best model as shown in Figure S4. X-tile was a published method for biomarker assessment and out-based cut-point optimization. X-tile can assess the robustness of the relationship between a biomarker and outcome by the construction of a two-dimensional projection of every possible subpopulation, which was also employed in other studies.[21,22] Using the optimal cut-off, Kaplan–Meier curve was generated in the test and complete sets to validate the used cutoff and risk score model.

Result

Description of Analytical Cohort

We obtained 69 tumor tissue samples from a total of 69 Asian patients with non-surgical esophageal cancer (AEC cohort, Table 1) was enrolled in this study with a median age of 64 years old (yrs), ranging from 41 to 83 yrs. More than 80% of the patients (81.60%) were male and the rest (17.39%) was female. Almost all patients were squamous cell carcinomas (SCC, 98.55%) except one patient was adenocarcinomas (ADC, 1.45%). There were 17 (24,64%) stage II, 40 (57.97%) stage III, and 12 (17.29%) stage IV patients. Among them, 65.22% of the patients were smokers and more than half of patients (52.17%) had a history of alcohol consumption. All AEC patients were treated with chemoradiotherapy.

Meanwhile, a TCGA cohort consisting of 143 patients with esophageal cancer was employed in this study (Table S1). The TCGA cohort included 46 (32.17%) Asian cases, 77 (53.84%) Caucasian cases, and 20 (13.99%) cases of other races. The median age was 61 yrs, ranging from 36 to 90 yrs. Similar to our cohort, the majority of the TCGA cohort was male (87.41%). Histology subtypes included 39.86% ADC and 60.13% SCC. The tumor stage included 78 (54.55%) stage II, 56 (39.16%) stage III, and 9 (6/29%) stage IV. In the TCGA cohort, 58.74% of the patients were non-smokers and 55.94% of the patients never had alcohol.

Table 1 Demographic Characteristics of Patients in AEC Cohort

Characteristics	AEC No. of Patients(%)
Total	69(100)
Race	
Asian	69(100)
Caucasian	0(0)
Others	0(0)
Age (years)	
≥65	29(42.03)
<65	40(57.97)
Median (range)	64(41–83)
Gender	
Male	57(81.60)
Female	12(17.39)
Histology	
ADC	1(1.45)
SCC	68(98.55)
TNM Stage	
Stage II	17(24.64)
Stage III	40(57.97)
Stage IV	12(17.29)
Smoking	
Positive	45(65.22)
Negative	24(34.78)
Alcohol	
Positive	36(52.17)
Negative	33(47.83)
Treatment history	
Chemoradiotherapy	69(100.00)

Genomic Landscape of Asian Patients with Esophageal Cancer

The genomic landscape of the AEC cohort and TCGA cohort was shown in Figures 1 and S1, respectively. In the AEC cohort, nearly 95% of patients harbored *TP53* alterations and more than half of patients were identified with *NOTCH1* alterations. Co-amplifications of *FGF19* and *CCND1* were found in 36.2% of cases. Other altered genes of high frequencies in the AEC cohort included *MCL1*(39.1%), *MYC* (31.9%), *PIK3CA* (21.7%), and *EP300*(18.8%). In the TCGA cohort, *TP53(85.3%)* and *PIK3CA (19.6%)* were the

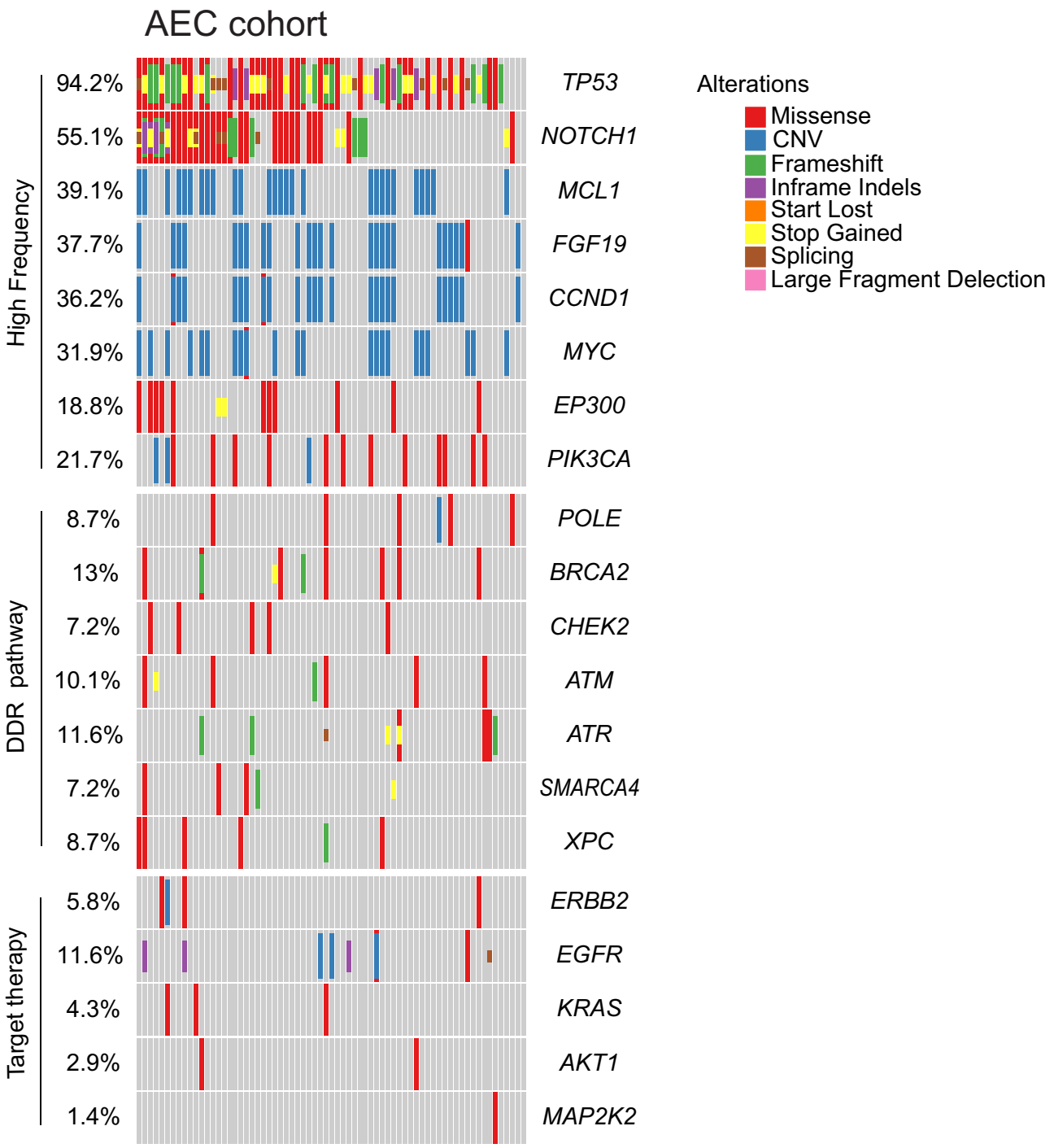

Figure 1 Genomic landscape of AEC cohort. The type of alterations was indicated by color. Each column represented one patient.

top two frequently mutated genes. Compared to the AEC cohort, co-amplifications of *FGF19* and *CCND1* were found at a slightly lower ratio (33.6%). Alterations in multiple DNA damage repair genes were identified in both cohort including *ATM* (10.1% vs 13.3%), *ATR* (11.6% vs 4.9%), *SMARCA4* (7.2% vs 6.3%). Interestingly, considering only targetable mutations, *EGFR* showed the highest ratio in the AEC cohort and *ERBB2* mostly amplification showed the highest ratio in the TCGA cohort.

Univariate and Multivariate Cox Regression Analyses of Prognostic Parameters

Clinicopathological features and genetic alterations are all potential predictors of prognosis in cancer treatment. Next,

we examined the association of these possible prognostic features with patients' overall survival (OS) using the univariate Cox regression model in the AEC cohort. As shown in Table 2, clinicopathological features including gender, age, smoking status, and alcohol consumption were not predictors of the OS in the AEC cohort. TNM stage showed some association with OS, with poorer outcome in stage III–IV patients compared to stage II (Figure S2). The most frequently observed alterations *TP53* mutation was not able to predict OS (Figure S3), which might due to limited *TP53* wild-type patients.

Interestingly, seven gene alterations were found to be independent markers of OS (Table 2) in the univariate model including *BAP1* mutation, *BRIP1* mutation, *KDR* mutation, *MYC* amplification, *RB1* variant (mutation and deletion), *WRN* mutation, *YAP1* amplification. In the AEC cohort, the frequencies of these seven gene alterations varied from 5.8% to 31.88% (Table 2). Next, we performed a multivariate cox regression analysis to predict OS using seven potential prognosis-related genes identified in univariate analysis (Table 3). Except for *KDR* with an insignificant p-value of 0.91, the rest six genes all showed as independent factors in multivariate analysis. Compared to wild type, patients with alterations in these genes were associated with poorer OS (Figure 2). Meanwhile, we also look into the association of *POLE* gene alterations and DDR pathway gene alterations with OS, however, the results were insignificant (Figure S4).

The Construction of Proportional Hazards Model with Six Prognosis-Related Genes and Evaluation

To distinguish EC patients with poor disease outcomes, a Cox proportional hazards model has been constructed using identified prognosis-related genes. The model was evaluated using a stepwise selection approach and the best model was then chosen with the combination of six

Table 2 Univariate Cox Regression Analyses of Prognostic Parameters

Characteristics	HR	95% CI	p value	Frequency (%)
Gender: Male (vs Female)	0.84	0.385–1.838	0.664	
Age: ≥ 65 yrs (vs <65 yrs)	0.76	0.404–1.420	0.385	
TNM Stage: III, IV (vs II)	1.80	0.849–3.835	0.120	
Smoking:Positive (vs Negative)	1.01	0.509–2.019	0.968	
Alcohol: Positive (vs Negative)	1.08	0.580–2.020	0.804	
TP53 mutation (vs Wild-type)	0.67	0.205–2.205	0.510	94.20
YAP1 CNV (vs Wild-type)	3.61	1.375–9.456	0.005	7.25
RB1 alteration (vs Wild-type)	3.03	1.248–7.348	0.01	11.59
BAP1 mutation (vs Wild-type)	4.12	1.226–13.866	0.013	5.80
MYC CNV (vs Wild-type)	1.88	0.994–3.538	0.049	31.88
BRIP1 mutation (vs Wild-type)	3.74	1.293–10.837	0.009	5.80
KDR mutation (vs Wild-type)	3.02	0.91–9.98	0.057	5.80
WRN mutation (vs Wild-type)	3.07	0.93–10.11	0.053	5.80

Note: Yrs, years old.

Table 3 Multivariate Cox Regression Analyses of Prognostic Parameters.

Characteristics	Univariate Analysis		Multivariate Analysis	
	HR(95% CI)	*p*	HR(95% CI)	*p*
YAP1 CNV (vs Wild-type)	3.606(1.375–9.456)	0.005**	4.061(1.450–11.370)	0.008**
MYC CNV (vs Wild-type)	1.875(0.994–3.538)	0.049*	2.187(1.048–4.566)	0.037*
RB1 alteration (vs Wild-type)	3.029(1.248–7.348)	0.010*	5.338(1.994–14.289)	<0.001***
BAP1 mutation (vs Wild-type)	4.123(1.226–13.866)	0.013*	5.131(1.435–18.349)	0.012*
BRIP1 mutation (vs Wild-type)	3.744(1.293–10.837)	0.009**	7.507(2.393–23.553)	<0.001***
KDR mutation (vs Wild-type)	3.020(0.914–9.983)	0.057	1.115(0.169–7.332)	0.910
WRN mutation (vs Wild-type)	3.066(0.93–10.11)	0.053	3.865(1.06–14.11)	0.041*

Note: *P<0.05; **P<0.01; ***P<0.001.

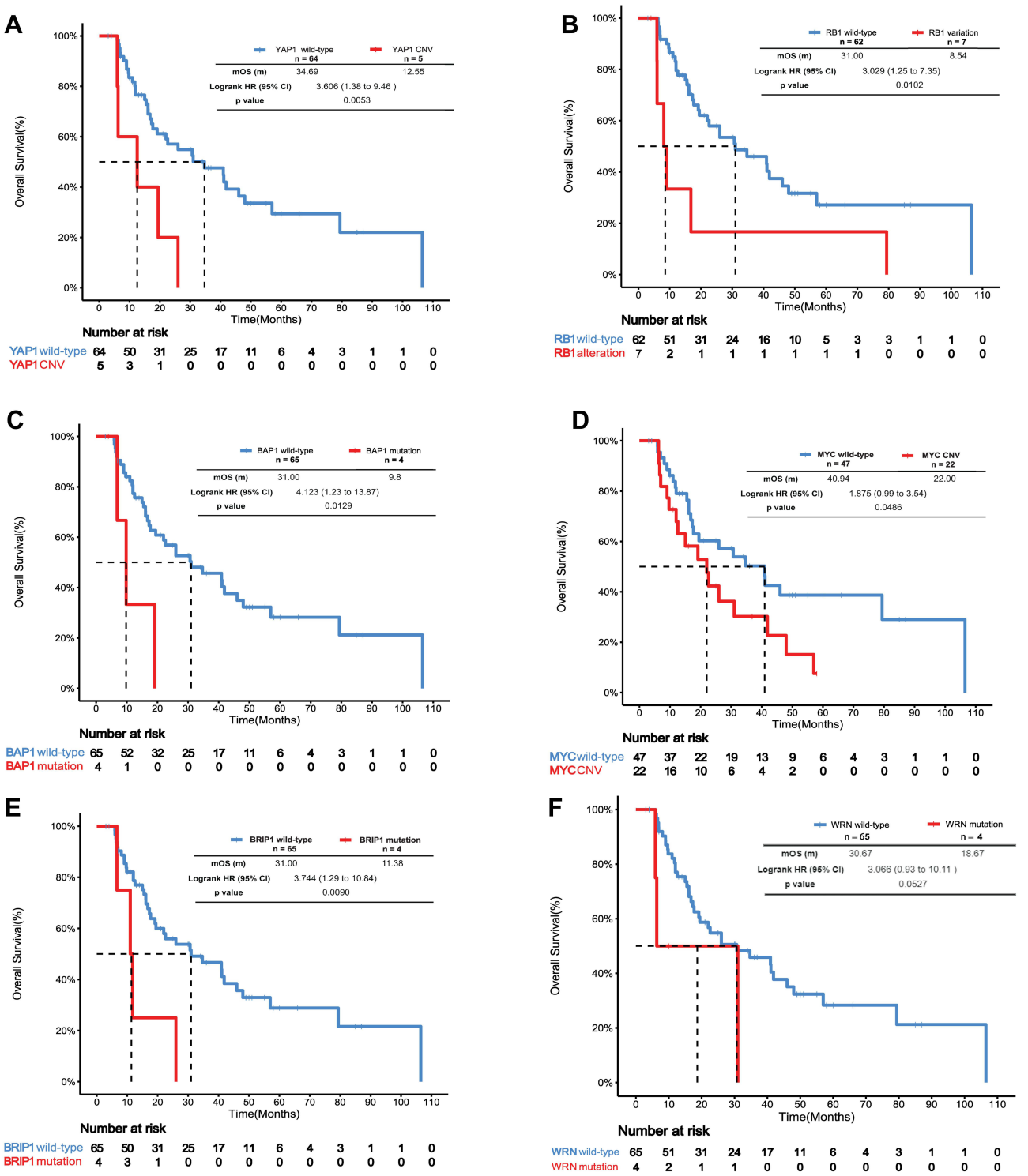

Figure 2 Survival analysis in AEC patients with different gene alterations. Kaplan–Meier survival curves for overall survival for the 69 EC patients from AEC cohort. The overall survival of patients with *YAP1* amplification (**A**), *RB1* alteration (**B**), *BAP1* mutation (**C**), *MYC* amplification (**D**), *BRIP1* mutation (**E**) and *WRN* mutation (**F**) was compared to that of patients with wild-type genes, respectively.

prognosis-related genes, which minimized the Akaike Information Criterion (AIC) and maximized index of concordance (C-index). The comparison of the C-index and AIC was shown in Table 4. The six-gene model achieved the lowest AIC of 264.09 and the highest C-index of 0.75 compared to models using single-gene alterations or other gene alteration combinations. The optimal cutoff point for risk score was set at 18 according to X-tile[22] (maximum

Table 4 Comparison of C-Index and Akaike Information Criterion (AIC) Between Models Using Different Variables

Included Variables	AIC	C-Index
BAP1 mutation	279.98	0.53
BRIP1 mutation	279.34	0.54
MYC CNV	280.08	0.57
RB1 variation	279	0.57
YAP1 CNV	278.59	0.55
WRN mutation	281.16	0.53
BRIP1 mutation + MYC CNV + RB1 variation +YAP1 CNV +WRN mutation	267.33	0.71
BAP1 mutation + MYC CNV + RB1 variation +YAP1 CNV +WRN mutation	270.79	0.71
BAP1 mutation +BRIP1 mutation + RB1 variation +YAP1 CNV +WRN mutation	264.76	0.7
BAP1 mutation +BRIP1 mutation + MYC CNV +YAP1 CNV +WRN mutation	270.79	0.69
BAP1 mutation +BRIP1 mutation + MYC CNV + RB1 variation +WRN mutation	268.67	0.73
BAP1 mutation +BRIP1 mutation + MYC CNV + RB1 variation +YAP1 CNV	265.29	0.74
BRIP1 mutation + MYC CNV +RB1 variation +YAP1 CNV +WRN mutation + KDR mutation	269.32	0.71
BAP1 mutation + MYC CNV +RB1 variation +YAP1 CNV +WRN mutation + KDR mutation	268.3	0.72
BAP1 mutation +BRIP1 mutation +RB1 variation +YAP1 CNV +WRN mutation + KDR mutation	266.7	0.71
BAP1 mutation + BRIP1 mutation + MYC CNV + YAP1 CNV + WRN mutation + KDR mutation	272.77	0.7
BAP1 mutation + BRIP1 mutation + MYC CNV + RB1 variation + WRN mutation + KDR mutation	270.67	0.73
BAP1 mutation + BRIP1 mutation + MYC CNV + RB1 variation + YAP1 CNV + KDR mutation	267.29	0.74
BAP1 mutation + BRIP1 mutation + MYC CNV + RB1 variation + YAP1 CNV + WRN mutation	264.09	0.75
BAP1 mutation + BRIP1 mutation + MYC CNV + RB1 variation + YAP1 CNV + WRN mutation + KDR mutation	266.07	0.75

χ^2, p<0.0001, Figure S5). Based on this cutoff, the Kaplan–Meier survival curves showed AEC patients with a risk score >18 (15 patients, median OS:10.40 months) displayed a poorer OS than a risk score<18 group (54 patients, median OS:41.86 months) (Figure 3A). We further examine the performance of this model using the TCGA cohort. The frequency of six prognosis-related genes in TCGA was shown in Table S2. With a cutoff at 18, this model was able to distinguish six EC patients with poor OS (median OS 14.31 vs 28.09 months, p=0.0008) (Figure 3B). We also validated our model using an independent Asian cohort of 47 ESCC patients treated with dCRT.[23] As shown in Figure S6, the model identified 22 ESCC patients with a risk score higher than 18, which displayed significantly worse OS (p=0.0022) than patients with a risk score lower than 18.

Discussion

Here, we employed a pan-cancer NGS panel of 422 genes to study the association between clinical characteristics, gene alterations, and overall survival in the Asian EC population. The prognostic value of six potential biomarkers was further evaluated in an EC cohort from TCGA. Compared to previous studies, the genomic landscape of this AEC displayed a higher ratio of *NOTCH1, MCL1, PIK3CA* alterations, which is likely due to the difference in the tumor stage as well as the sequencing method used.[11] In this AEC cohort, clinical characteristics including gender, age, smoking status, alcohol consumption were not associated with OS. Stage II EC patients showed slightly better OS than stage III–IV patients. Meanwhile, despite their high prevalence in EC, alterations in *TP53, PIK3CA,*

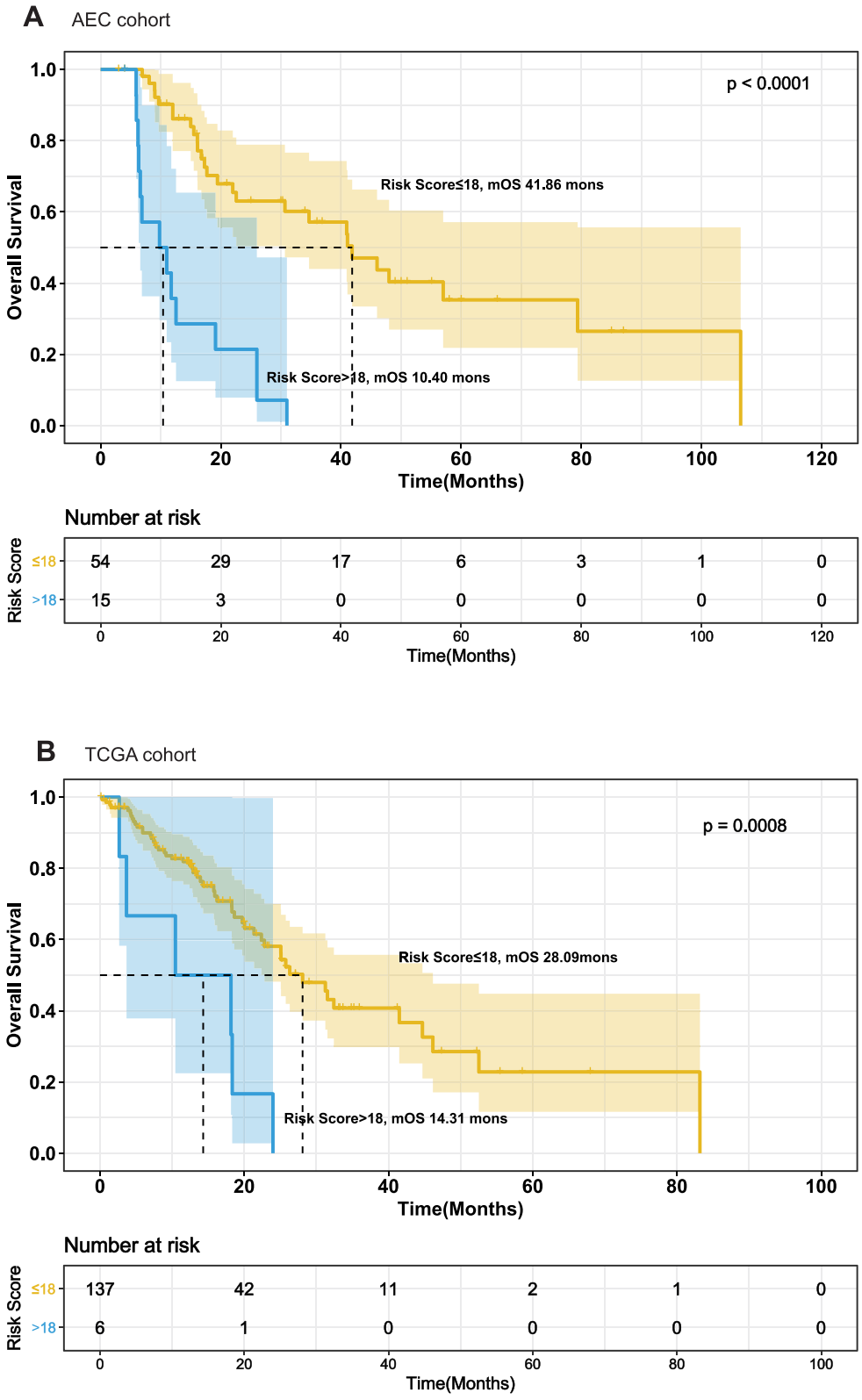

Figure 3 Kaplan–Meier curves for overall survival according to optimal cut-off point of six gene alterations in AEC (**A**) and TCGA (**B**) cohorts.

NOTCH1 as well as *FGF19* /*CCND1* co-amplification were not associated with OS, which is consistent with previous studies.[13,23]

Several gene alterations were identified as adverse prognostic factors in the AEC cohort, some of which belonged to different oncogenic signaling pathways in

cancers such as *YAP1* in the Hippo pathway, *MYC* in the MYC pathway, *RB1* in the cell cycle pathway.[24] *YAP1* amplification has been reported to be a prognostic factor of chemoradiotherapy in nonsurgical esophageal squamous cell carcinoma and is associated with shorter local recurrent-free survival and OS.[23] *MYC* amplification was also reported to be associated with poorer OS in ESCC patients.[25] The associations between the rest alterations and OS in EC were First reported in this study with some indications of their prognostic value in other cancer types. *BAP1, WRN*, and *RBIP1* are homologous recombination pathway-related genes, which can be targeted for DNA repair-targeted therapy.[26,27] *BAP1* is deubiquitylase associated with many cancer pathways and *BAP1* mutation has been reported as prognostic factors in predicting metastatic risk.[28,29] *BRIP1* and *WRN* encode RecQ DNA helicases and are important in the normal double-strand break repair.[30,31] Altered *BRIP1* is a targetable alteration in ovarian cancers and breast cancer patients with overexpression of *BRIP1* displayed a poor survival rate.[32,33] *KDR*, also known as *VEGFR-2*, plays an important role in tumor angiogenesis and potential therapeutic target for esophageal carcinoma.[34]

The use of genetic biomarkers in clinical settings is increasing, and many studies have been carried out on the characterization of prognostic biomarkers in EC patients to predict disease outcomes.[35–37] The identification of these markers provides a basis for detecting potential therapeutic strategies for specific molecular subtypes in clinical trials and will ultimately contribute to the personalized treatment plan for EC patients. For instance, some patients in AEC and TCGA cohort were identified as high-risk for poor disease outcomes using our model (a risk score higher than 18), different strategies could be considered for these patients: firstly, targeted therapy including trastuzumab (HER2 positive), ramucirumab (VEGFR); secondly, immunotherapy can be applied if the patient had MSI-H or PD-L1 expression according to NCCN guidelines;[7] Thirdly, as shown in Figure 1, altered DDR genes especially homologous recombination pathway genes such as *BRCA2, CHEK2, ATM/ATR* were also identified. Preliminary studies showed that PARP inhibitors may enhance the radiosensitivity in ESCC patients.[38,39] Therefore, PARP inhibitor together with radiotherapy may also be an option for high-risk populations. More studies are needed to validate our observation.

Major limitations of this study were the small cohort size and the lack of a large-scale Asian EC cohort to validate the six candidates for OS prediction. Most Asian EC studies were either RNA and gene expression level or the survival information of the cohort was unavailable. Here, we used a TCGA cohort, which consisted of 32.17% of Asian cases and 53.84% of Caucasian cases, whereas the AEC cohort was all Asian. Meanwhile, the distribution of histology subtypes varied in both cohorts. ESCC accounted for 60.13% of the TCGA and 97.1% of the AEC. Most of the treatment history from the TCGA cohort is unavailable, which may have some impact on the interpretation of the result. In conclusion, our study identified potential prognostic biomarkers for Asian EC patients. Further studies and validations of the prognostic value of these biomarkers in larger Asian clinical cohorts are warranted.

Abbreviations

EC, esophageal cancer; YAP1, yes-associated protein 1; RB1, retinoblastoma protein 1; BAP1, BRCA1-associated protein 1; MYC, MYC Proto-Oncogene; BRIP1, BRCA1 interacting protein C-terminal helicase 1; WRN, WRN RecQ Like Helicase; KDR, kinase insert domain receptor; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; NOTCH1, Notch homolog 1; FGF19, fibroblast growth factor 19; *CCND1*, cyclinD1 gene; ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3 related; SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4.

Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Availability of Supporting Data

All data that support the findings of this study are available from the corresponding authors upon a reasonable request.

Ethical Approval and Consent to Participate

Patient consent form was obtained from each patient following the guideline of Institutional Review Board requirements and the Declaration of Helsinki.

Consent for Publication

No individual data were used in this study.

Acknowledgments

We would like to thank the patients who participated in this study and their family, as well as the investigators and research staff involved.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

This study was supported by the Natural Science Foundation of Shandong (Grant No. ZR2020MH229) and the project of Shandong University (Grant No. 199/2019 heng).

Disclosure

Ruoying Yu, Jingwen Liu, Jiaohui Pang, Xue Wu and Yang Shao are shareholders or employees of Nanjing Geneseeq Technology Inc. The remaining authors have no conflicts of interest to declare.

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