

Multimic characterization of response and resistance to neoadjuvant chemoradiotherapy in esophageal squamous cell carcinoma

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Background: In neoadjuvant chemoradiotherapy (NCRT), only 1/3 of patients with esophageal squamous cell carcinoma (ESCC) achieve pathologic complete response (pCR). Here, we aimed to depict the biological landscape of ESCC with different responses to NCRT and identify biomarkers to facilitate clinical decision-making.

Methods: Tumor specimens before NCRT were obtained for whole exome sequencing (WES), RNA sequencing, and data-independent acquisition (DIA) mass spectrometry. Genomic data were analyzed for significantly mutated genes (SMGs), copy number alterations, microsatellite instability (MSI), tumor mutation burden (TMB), and mutational signatures. Transcriptomic and proteomic data were used to examine differentially activated pathways. Gene set enrichment analysis (GSEA) and ActivePathways were used for single omics and joint multimic analyses, respectively. Treatment-resistance biomarkers were identified and confirmed in a separate cohort using immunohistochemistry (IHC).

Results: FBXW7 mutation (Fisher's exact test, $P=0.03$) and 9p21.3 cytoband loss ($q\text{-value}=0.001$) are the significant genetic variations in the pCR group. Combined transcriptomic and proteomic analyses revealed that the type I interferon signaling pathways and retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) signaling pathways were enriched in non-pCR tumors. A biomarker panel of 12 proteins predictive of non-pCR tumors was identified, 10 of which were verified using multiplex IHC (mIHC) in an independent cohort.

Conclusions: We described the multimic biological characteristics of ESCC with distinct responses to NCRT and proposed a panel of proteins as predictive biomarkers for non-pCR patients.

Keywords: Genetic signature; multimics; treatment response; response biomarker; esophageal squamous cell carcinoma (ESCC)

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Introduction

Esophageal cancer ranks seventh in incidence and sixth in mortality rate according to the latest global cancer statistics (1). China accounts for approximately half of all esophageal cancer cases worldwide, with squamous carcinoma accounting for more than 90% of all cases. More than half of esophageal cancers are locoregional diseases at diagnosis (2,3). For resectable locally advanced esophageal cancer, neoadjuvant chemoradiotherapy (NCRT) followed by surgery is currently the standard of care. However, significant heterogeneity exists among patients with esophageal cancer. In esophageal squamous cell carcinoma (ESCC), only ~40% of patients achieve pathologic complete response (pCR) after NCRT, and in esophageal adenocarcinoma patients, the ratio is around 20% (4,5). Despite complete surgical resection, pCR patients appear to have superior survival outcomes and longer median time to recurrence than non-pCR patients (6). In order to improve the treatment outcome in esophageal cancer patients, it is imperative to elucidate the biological basis of NCRT resistance in esophageal cancer, as well as to find biomarkers for treatment-resistant patients to further individualize the treatment strategies, including delayed surgery and organ

preservation for pCR patients in ESCC (7,8). Previous and ongoing studies have reported different types of biomarkers for esophageal cancer NCRT response prediction, including gene polymorphisms and mRNA, miRNA, and protein markers. However, most previous studies were performed using microarray and immunohistochemistry, and studies taking advantage of next-generation sequencing to systemically discover biomarkers and differentially activated pathways are still lacking (9).

In this study, we performed multiomics sequencing on pre-treatment biopsied samples of ESCC and aimed to comprehensively depict the biological landscape of response and resistance to NCRT. Using whole exome sequencing (WES), transcriptomics, and proteomics data, we elaborated on both the genetic and environmental contributors to NCRT sensitivity and proposed biomarkers of NCRT resistance. We present this article in accordance with the MDAR reporting checklist (available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2026-1-0201/rc>).

Methods

Patient enrollment and sample processing

The recruited patients were histopathologically diagnosed with ESCC and received NCRT followed by esophagectomy at Shanghai Chest Hospital between 2016 and 2017.

Endoscopic biopsy samples of the primary tumor and paired peripheral blood samples were obtained from 24 patients before NCRT. The biopsied specimens were washed with PBS, transported in liquid nitrogen, and stored at -80°C until sequencing.

The NCRT regimen consisted of a total radiation dose of 41.4 Gy in 23 fractions, and the chemotherapy regimens consisted of fluorouracil and cisplatin. Surgery was performed within 10 weeks of NCRT, and tumor regression of the surgical specimen was evaluated by pathologists. pCR was defined as no sign of microscopically viable cancer cells in the primary tumor and lymph nodes encompassed in the radiation field.

The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. The study was reviewed and approved by the Shanghai Chest Hospital Institutional Review Board (IRB) (approval No. KS2040). The IRB determined that the requirement for informed consent was waived due to the retrospective nature of the study, in accordance with institutional and

Highlight box

Key findings

- Type I interferon and retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) pathway activation characterizes neoadjuvant chemoradiotherapy (NCRT)-resistant esophageal squamous cell carcinoma (ESCC). FBXW7 mutations and 9p21.3 IFN-cluster deletion are associated with chemoradiosensitivity. A 12-protein interferon-stimulated gene (ISG) biomarker panel predicts NCRT resistance.

What is known and what is new?

- Constitutive ISG expression is linked to radio/chemoresistance, but systematic multiomics profiling of NCRT response in ESCC has been lacking.
- This study integrates genomics, transcriptomics, and proteomics to connect FBXW7 loss and 9p21.3 deletion with IFN pathway downregulation and NCRT sensitivity, and proposes a validated 12-ISG protein biomarker panel for resistance prediction.

What is the implication, and what should change now?

- The biomarker panel could potentially enable pre-treatment stratification of ESCC patients to guide individualized treatment intensification and organ-preservation strategies with proper validation.

national ethical guidelines.

DNA extraction and WES

Genomic DNA was extracted from tumor tissues and paired peripheral blood samples using a TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Samples without blood controls were excluded from the study. DNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Inc., Wilmington, DE, USA), and the integrity was examined by 1% agarose electrophoresis. Exosomes were captured using Agilent SureSelect Human All Exon v6 library (Agilent Technologies, Santa Clara, California, USA) following the manufacturer's protocol. The DNA libraries were amplified and sequenced on an Illumina sequencing platform (NovaSeq 6000, Illumina, Inc., San Diego, CA, USA), and 150 bp paired-end reads were generated.

RNA extraction and RNA sequencing

RNA extraction was performed using TRIzol reagent (Invitrogen, Carlsbad, California, USA), and the integrity was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Samples showing significant degradation during nucleic acid electrophoresis were removed. Libraries were constructed using the VAHTS Universal V6 RNA-seq Library Prep Kit (Vazyme Biotech, Nanjing, China) according to the manufacturer's instructions and sequenced on the Illumina Novaseq 6,000 platform.

Data-independent acquisition (DIA) proteomics

Approximately 100 mg of frozen tumor samples from each patient were used for protein extraction using phenol extraction buffer. The total protein concentrations were measured, and the same quantity of protein from each sample was digested and desalted. Samples with an insufficient quantity of protein were removed. A Q-Exactive-HF mass spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Easy nanoLC-1200 (Thermo Fisher Scientific, Odense, Denmark) were used for both the shotgun proteomics and DIA experiments. A survey scan from 350 to 1,250 m/z at 35,000 resolution was adopted. Spectronaut Pulsar™ 15.3.210906.50606 (Biognosys, Schlieren, Zurich, Switzerland, Switzerland) search and library construction were performed using default settings. The DIA data were analyzed with Spectronaut, and the results were filtered based on a value cutoff of 0.01

[equivalent to false discovery rate (FDR) <1%].

WES data analysis

The raw data from paired tumor/blood samples were demultiplexed and converted into the FASTQ format. Raw reads were pre-processed using fastp (version 0.20.0) to obtain high-quality reads. Using Burrows-Wheeler Aligner (BWA), clean reads were aligned to the GRCh37 reference human genome (version 0.7.17). Based on the matched tumor and blood samples, MuTect (version 2.0) software was used to identify somatic single-nucleotide variant (SNV)/insertion-deletion mutation (INDEL) sites, and ANNOVAR software was used for annotation. With default parameters, MuSiC (version 0.4.1-1) further uses somatic variants (including SNVs and INDELs) to identify significantly mutated genes (SMGs). SMGs have a noticeably higher mutation rate than the background.

By using the Non-negative Matrix Factorization (NMF) method and aligning it with the known mutational signatures from the Catalog of Somatic Mutations in Cancer (COSMIC) database (10), the mutational signature of somatic SNV was retrieved. The purity and ploidy of the tumor samples were determined using a probabilistic model in Sequenza (version 2.1.2). Genomic Identification of Significant Targets in Cancer (GISTIC, version 2.0.23) was used to identify significant focal copy number variation (CNV) events and estimate log₂-transformed, gene-level, or segment-level CNV ratios. The GISTIC calls included -2 (deletion), -1 (loss), 0 (diploid), 1 (gain), and 2 (gain) (amplification).

The proportion of microsatellite instability (MSI) sites in each tumor sample was determined using MSIsensor software. The number of bases covered by the exome capture kit (34.3944 Mb) was divided by the total number of non-silent somatic SNVs and INDELs inside the exonic regions to determine tumor mutation burden (TMB).

For neoantigen analysis, individual human leukocyte antigen (HLA) typing was performed using Optitype from the WES data. For each non-synonymous SNV or non-frameshift INDEL, we built an amino acid FASTA sequence (mutant) with 23 amino acids, containing the mutated amino acid at position 12 and its corresponding wild-type peptides (normal). For frameshift mutations, the FASTA sequence was built from 12 amino acids upstream of the mutation towards the end of the transcript using the CCDS database from the National Center for Biotechnology Information (NCBI). IEDB netMHCpan 4.1 was used to

predict the HLA class I binding strength of each peptide. Mutant peptides with binding strength (IC_{50}) <500 nM and corresponding normal peptides with IC_{50} >500 nM were predicted as neoantigens. Neoantigens with fragments per kilobase per million (FPKM) >1 were defined as expressed.

RNA-seq data analysis

Fastp was used to first process raw reads in fastq format, removing any low-quality reads to provide clean reads. Using HISAT2, the clean reads were mapped to the reference genome. To evaluate the distribution of reads on the gene body, RseqQC was used. Because of the distribution bias on the 3' end of mRNAs, read counts within the 3'UTR region of each gene were obtained by HTSeq-count to ensure the accuracy of mRNA expression quantification.

Gene expression profile analysis

For RNA-seq data, FPKM was calculated to normalize gene expression. Differential expression analysis of transcriptomic data was carried out using DESeq2 (R package v1.38.3) and edgeR (R package v3.40.2). For proteomics data, limma (R package v3.54.1) and *t*-test were used for differential expression analysis.

To investigate the differentially activated cellular functional pathways in different response groups across transcriptomics and proteomics, the R package clusterProfiler was used. Gene set enrichment analysis (GSEA) was performed using the clusterProfiler R package (v4.18.4) with the fgsea algorithm. Genes were pre-ranked by log2 fold change from DESeq2 in decreasing order. Enrichment scores (ES) were calculated using a weighted running-sum statistic to assess whether predefined gene sets were overrepresented at the top or bottom of the ranked gene list. The ES was subsequently normalized to account for gene set size, generating a normalized enrichment score (NES) to allow comparison across gene sets. GSEA was conducted against Gene Ontology (GO) Biological Process gene sets (keyType = "SYMBOL") and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway gene sets (with gene symbols converted to Entrez IDs). Gene-set permutation was used with 1,000 permutations. Gene set size was filtered with a minimum of 10 and a maximum of 500 for GO analysis, and a minimum of 3 and a maximum of 800 for KEGG analysis. P values were adjusted using the Benjamini-Hochberg method. We also performed a

multiomics joint analysis using ActivePathways with the RNA and protein expression of all genes as input data (11). Adjusted P values were calculated using the Benjamini-Hochberg method.

Public dataset acquisition

The clinical information of 185 samples from The Cancer Genome Atlas (TCGA) Esophageal Carcinoma (ESCA) cohort was obtained from the TCGA data portal, and 36 out of 185 ESCC samples with recorded radiotherapy responses were chosen for biomarker validation analysis (data accession https://www.cbiportal.org/study/summary?id=esca_tcga; data source: TCGA Esophageal Carcinoma, Firehose Legacy).

ESCC cell culture and treatment

TE-1 and KYSE510 ESCC cell lines were selected based on their reported differences in intrinsic radiation response characteristics. Preliminary clonogenic survival assays confirmed that TE-1 exhibits relatively higher baseline radiosensitivity, whereas KYSE510 displays comparatively greater radioresistance. These two cell lines were therefore used to model biologically distinct ESCC phenotypes in subsequent functional experiments.

The human ESCC cell line TE-1 (RRID:CVCL_1759) and KYSE510 (RRID:CVCL_1354) were purchased from the Cell Bank/Stem Cell Bank, Chinese Academy of Sciences. Both cell lines have been authenticated using short tandem repeat (STR) profiling within the last 3 years. All cellular experiments were performed with mycoplasma-free cells.

TE-1 and KYSE510 cells were cultured in RPMI1640 medium (Cytiva, Marlborough, Massachusetts, USA) with 10% FBS (Gibco, Life Technologies Inc., Carlsbad, California, USA) at 37 °C in 5% CO₂/95% air.

Cell radiation treatment: when growing up to 70–80% confluence, ESCC cells were irradiated using the Small Animal Radiation Research Platform (SARRP, Xstrahl Inc., Suwanee, Georgia, USA) at a dose rate of 2.4 Gy/min.

Interferon treatment: TE-1 was subjected to a 16-day continuous low-dose IFN- β (Chamot Biotechnology Co., Ltd., Shanghai, China) stimulation regimen (5 IU/mL), with media replacement every other day, and the addition of fresh IFN- β .

Apoptosis will be detected through staining of the cells with Annexin V and propidium iodide (PI) solution

(LIANKE Bio., Hangzhou, China), followed by flow cytometry analysis. Each experiment was repeated 3 times.

FBXW7 gene knockdown and IFN level measurement

FBXW7 gene knockdown was accomplished by employing siRNA with the following sequence: Sense (5'-3'): CGGGUGAAUUUAUUCGAAATT; Antisense (5'-3'): UUUCGAAUAAAUUCACCCGTT. Human ESCC cell line KYSE510 was used for the gene knockdown. KYSE510 cells were seeded to reach a confluence of approximately 70% before transfection. The transfection reagent used was GP-transfect-Mate (GenePharma, Shanghai, China), with the siRNA working concentration of 100 nmol/L. In the gene knockdown group, transfection was executed with a mixture of transfect-mate (5 μ L in 1 mL) and siRNA (100 nM), while the control group received transfect-mate exclusively. The transfection efficiency was evaluated using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Intracellular type I IFN levels were quantified using IFN- α and IFN- β enzyme-linked immunosorbent assay (ELISA) kits (XY-biotechnology, Hangzhou, China). IFN level assessment was conducted 48 h after the siRNA transfection. Treated cells were subjected to freeze-thaw cycles to induce cell lysis, and the supernatant was collected for measurement of IFN- α and IFN- β levels.

Immunofluorescence

Immunofluorescence was performed for STAT1, EIF2AK2, MX1, BST2, TRIM21, SAMHD1, IFI44L, GBP1, PARP14, ISG15, HLA-B, and IFIT3, and the tumor cells were stained with P63. The TSAPlus fluorescent multiplex immunohistochemistry (mIHC) kit (Wuhan Servicebio Technology Co., Ltd., Wuhan, China) was used for antigen staining. The antibodies used are listed in Table S1. The stained sections were scanned using Panoramic DESK (3DHISTECH Ltd., Budapest, Hungary).

Statistical analysis

Statistics were performed using R (version 4.0.4). Unless otherwise specified, the continuous variables and categorical variables are compared with the Student *t*-test and Chi-squared test; Benjamini and Hochberg FDR procedure was employed to adjust P values for multiple comparisons; tests were two-sided, and a P value or an adjusted P value of

less than 0.05 was considered significant. The correlation analysis was performed by calculating the Spearman or Pearson correlation coefficient.

The joint analysis of transcriptomics and proteomics data was performed using ActivePathways in R. The significance of the enriched pathways across multiomics data was evaluated by hypergeometric P value, which was corrected as Q_{pathway} using the Holm-Bonferroni method of family-wise error rate (FWER) (11). Pathways with $Q_{\text{pathway}} < 0.05$ were reported as significant.

SMGs in two response groups were analyzed using Fisher's exact test. The TMB differences were analyzed using the Mann-Whitney *U* test. In CNV analysis, the adjusted P value using the Benjamini and Hochberg FDR procedure was calculated by the GISTIC2.0 package and reported as q-value.

Kaplan-Meier plotter (kmplot.com/analysis/) was used to evaluate the correlation of the biomarker panel expression level and the survival outcomes of ESCC patients, which utilizes gene expression data sources including TCGA, EGA, and GEO, etc. (12).

Results

Patient information and quality control of samples

Among the 24 patients, 13 achieved pCR (54%). The baseline clinical characteristics, including sex, age, smoking history, tumor location, tumor clinical T stage, and N status, were similar between the two response groups (Table S2).

WES was performed on 18 tumor samples, as six lacked a blood control. Seven RNA samples showed significant degradation on electrophoresis, and one sample contained insufficient protein for spectrometry. These samples were not included in the experiment. In total, RNA sequencing and DIA spectrometry were performed on 17 and 23 samples, respectively (Table S2).

Interferon signaling and retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) signaling pathways were enriched in non-pCR tumors in the joint analysis of transcriptomics and proteomics

RNA-seq was performed in 17 of the 24 patients. Genes differentially expressed between pCR and non-pCR patients were analyzed using EdgeR and DESeq2 in R. The 85 genes at the intersection of the two results were identified as differential genes ($P < 0.005$, $\log_2\text{FC} > 0.58$ or

<-0.58) (Figure S1, Table S3). GSEA analysis highlighted that the most significantly enriched pathways in the non-pCR group were the type I interferon signaling pathway and the defense response to viruses (adjusted P value =0.000). Conversely, the pCR patients showed significant enrichment in keratinization-related pathways (adjusted P value <0.001) (Figure 1A and Table S4). Furthermore, in non-pCR patients, the MDA-5 signaling pathway, an upstream regulator of interferon signaling, was enriched, as revealed by GO enrichment analysis of the differential genes (adjusted P value =0.05).

DIA mass spectrometry was performed in 23 of the 24 patients, identifying 4,080 proteins. Analysis using principal component analysis (PCA) and unsupervised hierarchical clustering with valid proteins revealed distinct protein expression patterns in patients with distinct treatment responses (Figure S1). Combined limma and *t*-test identified 385 differentially expressed proteins ($P<0.05$, $\log_2FC >0.58$ or <-0.58) between pCR and non-pCR patients (Figure S1). GO enrichment analysis was performed based on the differential proteins, and the top 30 enriched pathways were all derived from the non-pCR group (Figure 1B). The top enriched pathways included RNA catabolic-related and neutrophil activation pathways.

In accordance with the transcriptomic enrichment results, the type I interferon signaling pathway was also among the significant pathways enriched in the non-pCR group (adjusted P value =0.003). In addition, the dsRNA response pathway and Wnt signaling pathway enriched in proteomics corresponded with the MDA-5 signaling pathway and Wnt protein secretion pathway in transcriptomics, respectively (Figure 1C).

To identify the consistently enriched pathways across different gene expression omics while also detecting pathways not apparent in any single-omic dataset, we integrated the transcriptomics and the proteomics data using the ActivePathways package in R (11). In genes elevated in the non-pCR group, the joint analysis of ActivePathways highlighted 315 genes that were significantly enriched in 165 pathways (Figure 1D). Approximately 20% of the enriched pathways were supported by data from both RNA and protein levels, and 24% of the pathways were identified by multiomics joint analysis. Notably, the type I interferon signaling and interferon regulatory pathways were frequently observed in these two categories. Additionally, our analysis highlighted the significant enrichment of the upstream pathway of interferon, the “RIG-I signaling pathway”, in the non-pCR group ($Q_{\text{pathway}}=0.015$).

Both RIG-I and MDA5 are members of RLRs. RLRs

are RNA sensors localized in the cytosol and could induce type I interferon production in the detection of unusual nucleic acids (13). Our data support that the activation of both the RLR pathway and its downstream interferon signaling pathway contributes to NCRT treatment resistance in ESCC. It's well established that the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway is the predominant downstream signal transduction pathway after interferon signal activation (14). In our differential expression gene analysis, STAT1 was significantly up-regulated in non-pCR group in both transcriptomics data (P value =0.001, FC =0.43) and proteomics data (P value =0.03, FC =0.48). Our findings are corroborated by previous research, which revealed that RIG-I-induced STAT1 activation leads to chemotherapy and radiotherapy resistance in breast cancer (15).

Genetic characteristics of patients receiving NCRT with different responses

A total of 18 biopsied samples were subjected to WES with the corresponding blood samples as controls, among which 9 samples (50%) were pCR after NCRT.

First, we evaluated the general genetic differences between the pCR and non-pCR groups (Figure 2). TMB, described as mutations (SNVs or INDELs) per megabase, was not significantly different between groups [pCR: median 10.32, standard deviation (SD) 3.63, interquartile range (IQR) 4.84; non-pCR: median 9.36, SD 4.75, IQR 3.05; Mann-Whitney *U* test, $P=0.84$]. The differences in other genetic variations (Table S5), including total somatic copy number alteration (CNA) length (*t*-test $P=0.61$ for copy number gain, $P=0.39$ for copy number loss), somatic variation (SV) counts (*t*-test $P=0.36$), MSI (*t*-test $P=0.73$), and tumor purity (*t*-test $P=0.89$), were not statistically significant between the two response groups. Gene mutation signatures were investigated via nucleotide mutation signature and the COSMIC database v3 (16). The results revealed that the most common nucleotide mutations were C>T and T>C among all samples; there was no nucleotide mutational difference between the two groups (Figure S2). The COSMIC signatures SBS25 and SBS9 were detected in all patients, and SBS9 was more frequently detected in the pCR group (Figure S2). We further analyzed the differences in neoantigen load between the groups. Neither neoantigen load nor expressed neoantigen load was associated with the pathological response (Figure S3).

Gene mutations

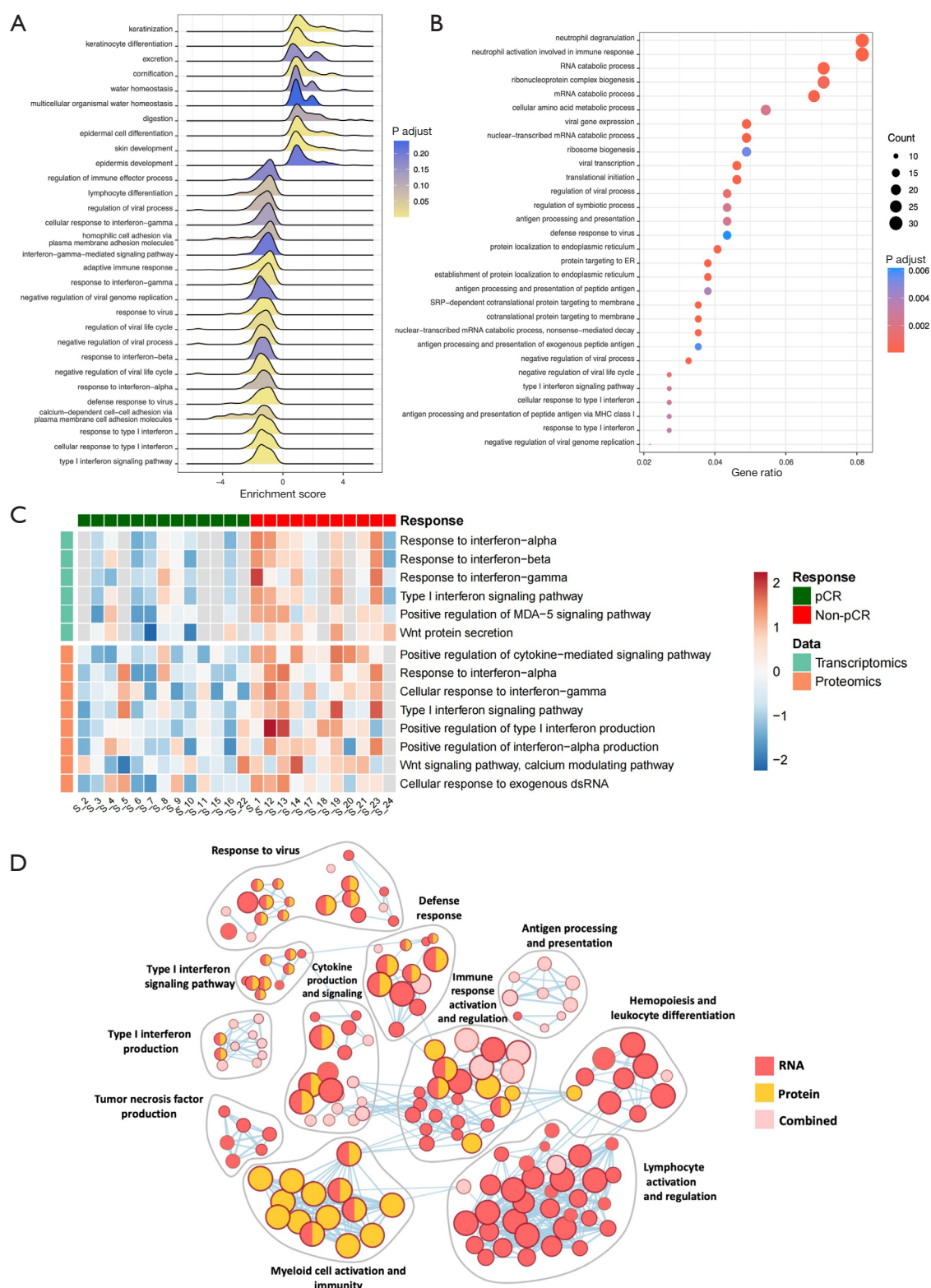


Figure 1 Differential gene expression and pathway enrichment at the transcriptomics and proteomics levels. (A) RNA-seq data were used in GSEA pathway enrichment analysis (enrichment in the pCR group, enrichment score >0; enrichment in the non-pCR group, enrichment score <0). The top 30 enriched pathways are presented. (B) GO enrichment analysis of the differential proteins (DIA mass spectrometry data

was used for analysis). The top 30 differential pathways were enriched in the non-pCR group. (C) Selected corresponding pathways enriched in transcriptomics and proteomics. The color scale represents the NES, where red indicates positive enrichment and blue indicates negative enrichment. (D) ActivePathways enrichment map showing pathways enriched in transcriptomics, proteomics, and omics joint analysis. Nodes represent pathways annotated by GO. Magenta coloring represents evidence from transcriptomics; orange coloring represents evidence from proteomics; pink coloring represents evidence from joint omics. DIA, data-independent acquisition; ER, endoplasmic reticulum; GO, Gene Ontology; GSEA, gene set enrichment analysis; MHC, major histocompatibility complex; NES, normalized enrichment score; pCR, pathologic complete response; SRP, signal recognition particle.

Next, we performed an SMG analysis of the two response groups. TP53 was the most mutated gene with a mutation frequency of 72% across all samples (Figure 3A). *FBXW7* was the only gene with significantly different mutation frequencies between the groups (Fisher's exact test P value =0.03), with five out of nine pCR patients carrying non-synonymous mutations, in contrast to no mutation in the non-pCR group. Mutation types included missense and frameshift mutations in the F-box, COG2319, and WD40 domains (Figure 3B).

In a previous study, Gstalder *et al.* demonstrated that inactivation of *FBXW7* in a melanoma model was associated with impaired tumor-intrinsic expression of the dsRNA sensors MDA5 and RIG-I, and thus decreased induction of type I interferon expression, which leads to anticancer therapy resistance (17). Given the observed differences in RLR and type I interferon signaling pathway expression levels between pCR and non-pCR patients, we sought to investigate if these expression variations were linked to *FBXW7* mutation status. To do so, we conducted a differential gene expression and enrichment analysis, comparing samples with *FBXW7* mutations to those with *FBXW7* wild-type status using transcriptomic data. The results of GSEA revealed a notable reduction in the expression of the type I interferon signaling pathway in *FBXW7*-mutated samples (Figure 3C,3D). Meanwhile, GO enrichment analysis showed that the interferon upstream RLR pathways, including the MDA-5 signaling pathway (adjusted P value =0.002) and the RIG-I signaling pathway (adjusted P value =0.018), were both downregulated in *FBXW7*-mutated samples by GO enrichment analysis (Table S6). RNA expression data revealed positive correlations between *FBXW7* expression and key RLR pathway genes [Spearman correlation coefficient: IFIH1, $r=0.602$; DDX58 (RIG-I), $r=0.400$; TBK1, $r=0.694$], as well as type I interferon-induced JAK-STAT pathway key gene expression (Spearman correlation coefficient: STAT1, $r=0.449$; JAK1, $r=0.588$; TYK2, $r=0.306$) (Figure 3E) in

17 samples that underwent RNA-seq. Moreover, we analyzed the TCGA ESCC dataset (esophageal carcinoma, TCGA, Firehose Legacy) on cBioPortal (18), and verified the co-expression tendency of *FBXW7* with key genes in the RLR and JAK-STAT pathways (measured by Spearman correlation coefficient, Table S7).

In addition to its regulatory effect on RLR and interferon signaling, *FBXW7* was also reported to contribute to DNA repair via nonhomologous end-joining and recovery of the cell cycle by promoting P53 degradation after DNA-damaging therapies. Inactivation of *FBXW7* enhanced tumor radiosensitivity and chemosensitivity (19). The GO enrichment analysis in *FBXW7* mutated samples in our cohort exhibited a significantly lowered expression in the "DNA damage response" pathway (adjusted P value =0.002).

Collectively, our data suggest that inactivation of *FBXW7* in ESCC is sensitive to NCRT. It might exert an effect on the treatment response through the regulation of interferon-related pathways and DNA damage response pathways.

CNVs

CNVs were investigated in all samples using GISTIC (Figure S4). In the non-pCR group, the most significant CNVs were 20q13.33 amplification (q-value =0.000) and 4q35.2 deletion (q-value =0.044). In the pCR group, 8q24.3 (q-value =0.000) was the top amplified cytoband, encompassing genes that encode a series of microRNAs and oncogenes such as *PARP10*. The most significant cytoband loss in the pCR group was 9p21.3 (q-value =0.001), which is a 917kb long chromosome band containing *CDKN2A/B*, *MTAP*, and a type I IFN gene cluster (Figure 3F,3G). Loss of chromosome 9p21.3 has been reported as the most common homozygous deletion in human cancers. While 9p21.3 deletion almost always involves *CDKN2A*, co-deletion of the IFN gene cluster only occurs in certain patients, which disrupts the tumor microenvironment and changes the

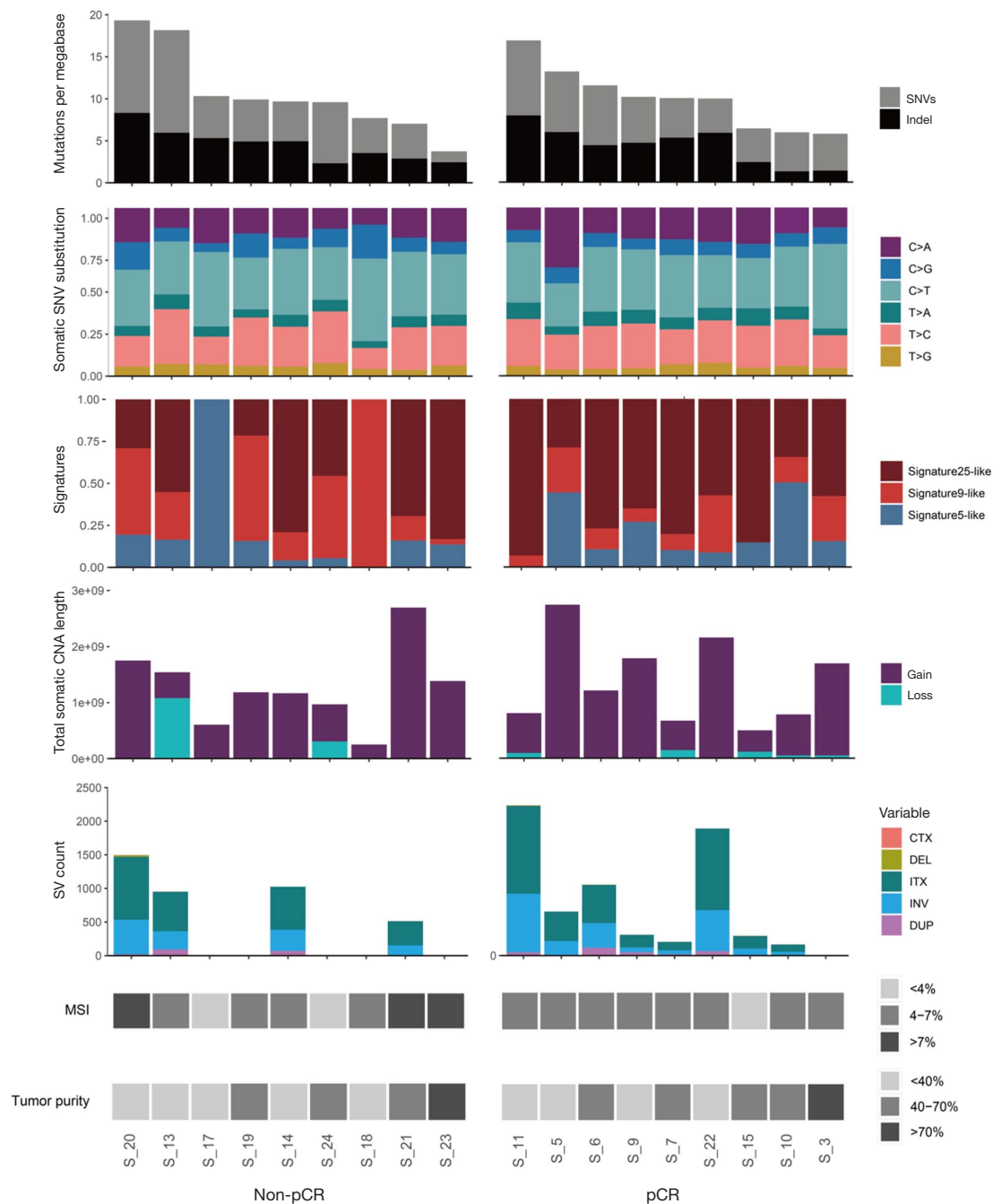


Figure 2 Genetic landscape. The plot displays the overall genetic landscape of pCR tumors compared to non-pCR tumors. TMB includes SNVs and INDELs. TMB was similar between response groups (Mann-Whitney U test, $P=0.84$). Gene mutation signatures analysis included single-nucleotide substitution signatures (second line) and COSMIC signatures (third line). Total somatic I length (t -test $P=0.61$ for copy number gain, $P=0.39$ for copy number loss), SV counts (t -test $P=0.36$), MSI (t -test $P=0.73$), and tumor purity (t -test $P=0.89$) were not statistically significant between the two response groups. CNA, copy number alteration; COSMIC, Catalog of Somatic Mutations in Cancer; CTX, chromosomal translocation (specifically inter-chromosomal translocation); DEL, deletion; DUP, duplication; INDELs, insertion-deletion mutations; INV, inversion; ITX, intra-chromosomal translocation; MSI, microsatellite instability; pCR, pathologic complete response; SNVs, single nucleotide variations; SV, somatic variation; TMB, Tumor mutation burden.

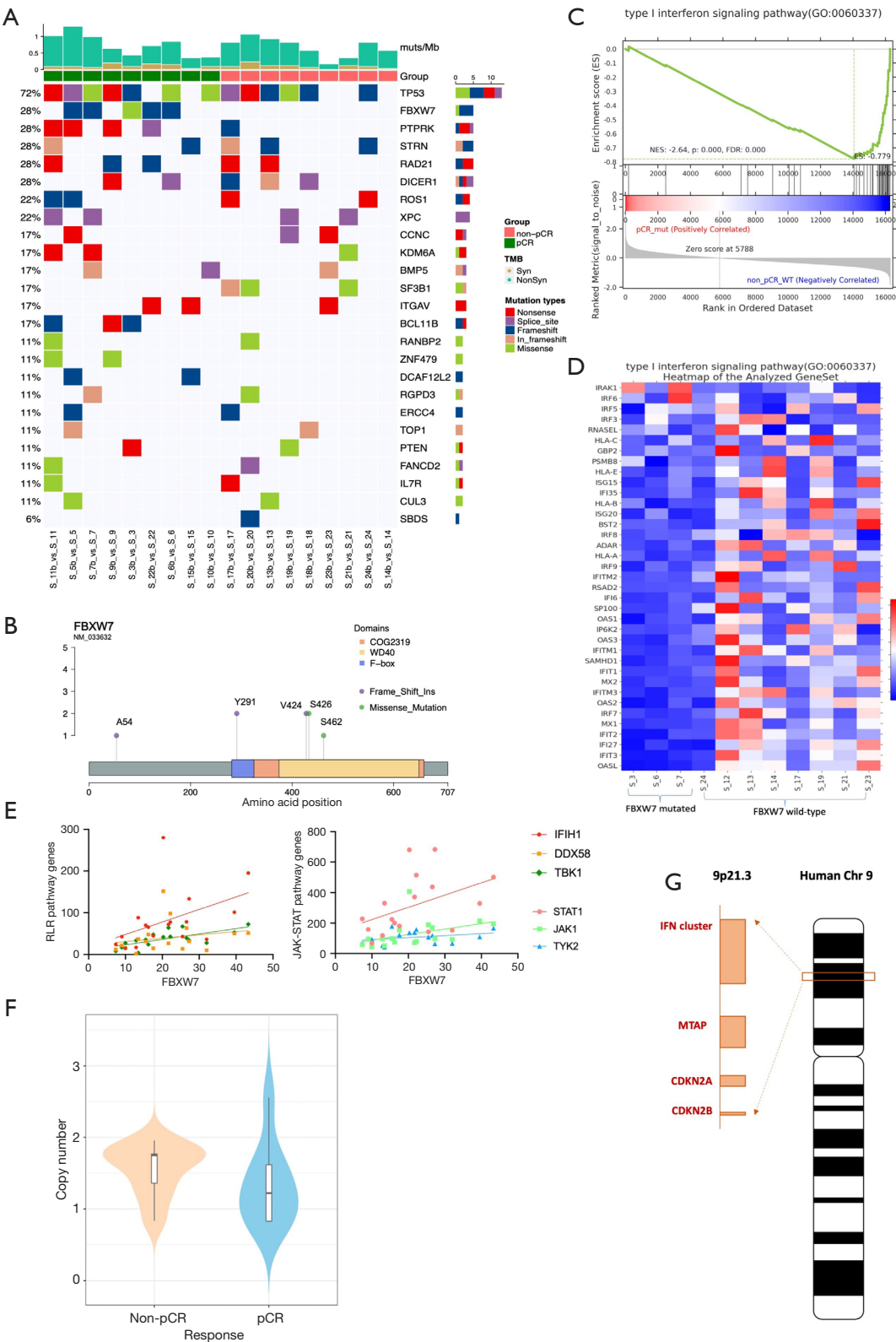


Figure 3 Gene mutations and copy number variation analysis. (A) Gene mutation plot. The top bar of the plot shows the mutation burden of each sample, partitioned by Syn and NonSyn. The major part of the plot displays the top mutated genes in each sample. The left

column shows the mutation frequency of each gene. (B) Types of FBXW7 gene mutations detected in WES in our pCR cohort. The y-axis represents the mutated sample number. (C) GSEA enrichment analysis using transcriptomic data shows the type I interferon signaling pathway is enriched in FBXW7 wild-type samples and has decreased expression in FBXW7 mutated samples (which are also pCR samples). (D) The gene expression heatmap of the type I interferon signaling pathway in FBXW7-mutated versus wild-type samples. (E) FBXW7 expression level is positively correlated with key gene expression levels from the RLR pathway and the JAK-STAT pathway. (F) 9p21.3 cytoband copy number difference between the pCR and non-pCR groups. (G) Schematic view of cytoband 9p21.3 and the genes mapped on this region. The IFN cluster contains an array of type I interferon genes. ES, enrichment score; FDR, false discovery rate; GO, Gene Ontology; GSEA, gene set enrichment analysis; JAK-STAT, Janus kinase-signal transducer and activator of transcription; NES, normalized enrichment score; NonSyn, non-synonymous mutation; pCR, pathologic complete response; RLR, retinoic acid-inducible gene I-like receptor; Syn, synonymous mutation; TMB, Tumor mutation burden; WES, whole exome sequencing.

tumor biological behaviors in these patients (20).

In our pCR cohort, GISTIC results showed that five out of nine pCR patients carry 9p21.3 CNVs, including CDKN2A deletion and co-deletion of IFN genes. We presume that the chromosome 9p21.3 IFN cluster deletion may partially contribute to the relatively lower expression of the type I IFN signaling pathway in the pCR group at both the transcriptomic and proteomic levels. In the ESCC TCGA cohort (esophageal carcinoma, TCGA, Firehose Legacy) from cBioPortal, 51 out of 96 patients carry a 9p21.3 deletion, among which 13 patients had co-deletion of IFN genes. Initially, we found no significant difference in the expression level of the interferon signaling pathway between patients with and without non-IFN cluster 9p21.3 copy number deletion. However, when comparing patients with and without 9p21.3 deletions that included the IFN cluster, we observed a significant increase in the expression of RIG-I, IFN signaling, and JAK-STAT pathways in the copy number deletion group (Figure S4). Thirty-six patients in the TCGA ESCA cohort had recorded response evaluation of radiotherapy, among whom three patients were found to carry 9p21.3 IFN cluster deletion, and all of their radiotherapy responses were complete remission.

The findings in the TCGA cohort reaffirmed our assumption that chromosome 9p21.3 copy number deletion with IFN-cluster is a sensitive genetic variance that enhances ESCC NCRT treatment response, and it might exert its pro-therapeutic effect by downregulating interferon signaling in the tumor cells.

Assessment of the IFN effect on ESCC cell line radiosensitivity

Previous studies have reported that constitutive elevation of type I IFN was seen in some cancers (21). And the chronic exposure to low-dose type I IFN may promote cell

resistance to DNA damage through elevated expression of a subset of IFN-induced genes, including STAT1, STAT2, and IRF9 (22). To investigate whether chronic exposure to type I IFN promotes radiotherapy resistance of ESCC, we treated the ESCC cell line TE-1 with low-dose IFN- β (5 IU/mL) continuously for 16 days. The treated cells and the untreated control received irradiation of 0, 2, 4, and 8 Gy, and apoptotic percentages were measured 48 h after radiation.

In comparison to the control group, TE-1 cells treated with 5 IU/mL IFN- β for 16 days exhibited a significantly reduced number of apoptotic cells following exposure to 2 Gy (t -test $P=0.03$), 4 Gy (t -test $P=0.02$), and 8 Gy (t -test $P=0.01$) radiation (Figure 4A,4B), indicating increased radioresistance after low-dose IFN- β treatment. The results supported the hypothesis that the primary radioresistance observed in NCRT treatment may derive from the constitutive activation of type I IFN signaling in ESCC.

Knockdown of FBXW7 alters intracellular IFN levels

To evaluate the effect of FBXW7 loss on type I interferon expression in ESCC, we performed siRNA-mediated knockdown of FBXW7 in the KYSE510 cell line. A specific siRNA sequence (FBXW7-Homo-1470) was used for subsequent experiments. Knockdown efficiency was validated at both the mRNA and protein levels. At 48 h post-transfection, FBXW7 mRNA expression was reduced to approximately 42% of control levels as determined by qRT-PCR, and protein expression was reduced to approximately 46% of control levels as assessed by Western blot densitometric analysis. The intracellular levels of both IFN- α and IFN- β were significantly decreased in the FBXW7-siRNA group compared with the control group (IFN- α , $P=0.000$; IFN- β , $P=0.01$) (Figure 4C). This result supports our hypothesis that the loss-of-function mutation

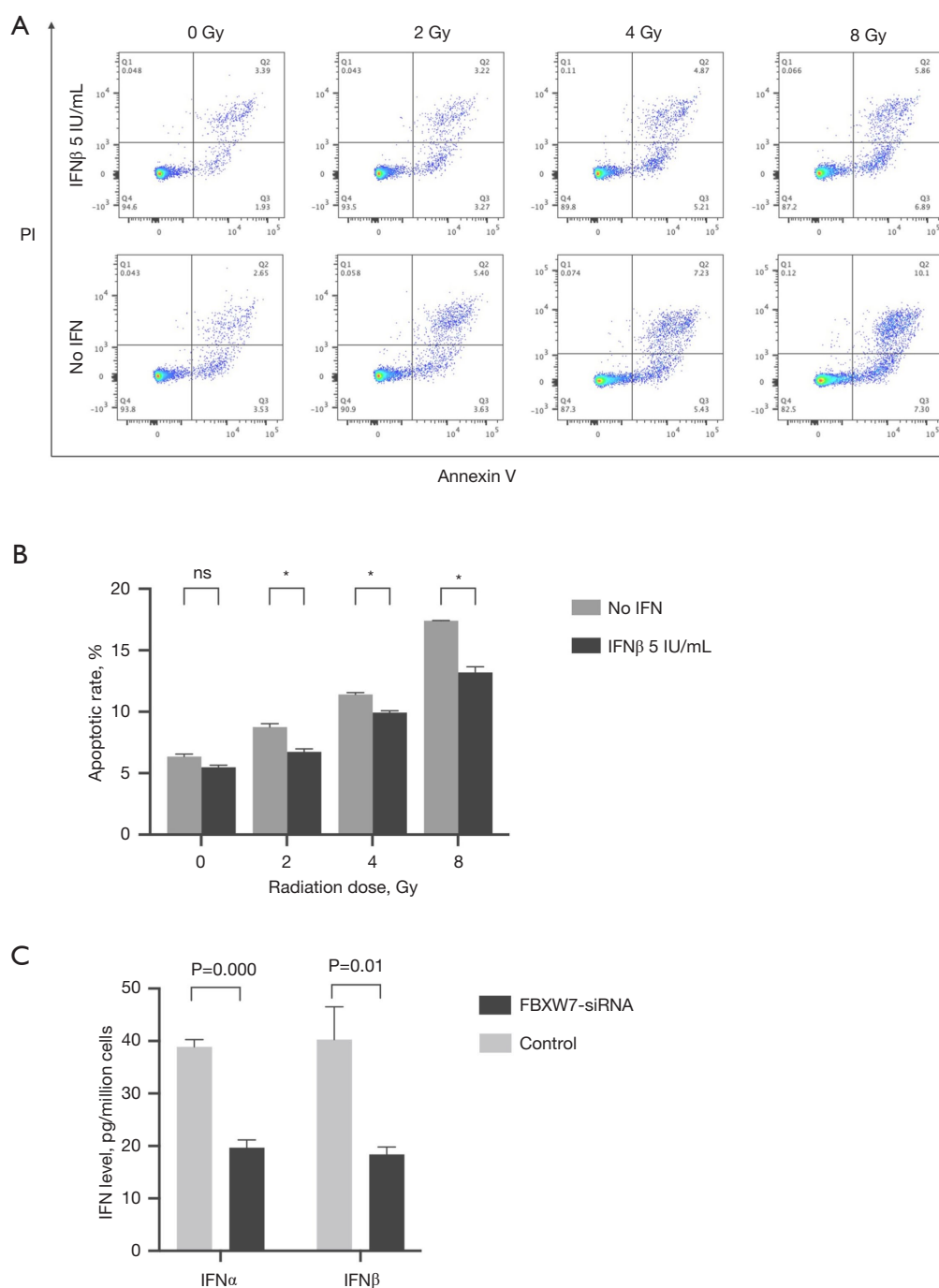


Figure 4 *In vitro* assessment of IFN effects on radiosensitivity and FBXW7 KD effect on IFN level. (A,B) Esophageal squamous cell cancer cell line TE-1 was treated with low-dose (5 IU/mL) IFN- β continuously for 16 days and received radiotherapy. Cell apoptotic rates were measured 48 h after radiation. Cells treated with IFN- β show increased radiation resistance in 2, 4, and 8 Gy radiation doses. Comparisons were made using the *t*-test: 0 Gy ($P=0.07$), 2 Gy ($P=0.03$), 4 Gy ($P=0.02$), and 8 Gy ($P=0.01$). Error bars show SEM. (C) FBXW7 siRNA knockdown lowers KYSE510 intracellular type I IFN levels. Intracellular IFN- α and IFN- β levels were measured by ELISA assay 48 h after transfection. The control group receives transfer-mate only. Error bars show SEM. *, $P<0.05$. ELISA, enzyme-linked immunosorbent assay; ns: not significant; PI, propidium iodide; SEM, standard error of the mean.

of FBXW7 could lower the constitutive expression level of type I IFN in ESCC, and may further alter the radiosensitivity through this effect.

Interferon-stimulated genes (ISGs) as resistance biomarkers of ESCC patients treated with NCRT

After NCRT, patients with pathological complete remission have distinct gene expression profiles at the protein level compared with non-pCR patients. We aimed to define a protein biomarker panel that can identify NCRT-resistant tumors. Protein-protein interaction (PPI) analysis was performed using 385 differentially expressed proteins in the two response groups. Next, the genes in the PPI network were ranked based on their topological score calculated using the cytoHubba MCC method (23). Forty-five hub genes were identified at a cutoff score of 50,000. The hub genes were further selected using transcriptomic data, and the final protein biomarkers were defined as the intersection of PPI hub genes and transcriptomic differential expression genes (DESeq2, P value <0.05) (Table S8). The biomarker panel comprised 12 proteins: STAT1, EIF2AK2, MX1, BST2, TRIM21, SAMHD1, IFI44L, GBP1, PARP14, ISG15, HLA-B, and IFIT3. All proteins had relatively elevated expression in the non-pCR group; thus, the panel could identify ESCC patients with potential NCRT resistance. The corresponding genes of these proteins were all members of the ISG family, and active interactions were observed within their PPI network (Figure 5A) (24).

The 12-protein biomarker panel was tested in the 36-patient subset with a recorded radiotherapy response from the TCGA ESCA cohort (Table S9). GSEA demonstrated significant enrichment of the biomarkers in non-complete response (radiographic progressive disease, stable disease) patients (NES, -1.852; adjusted P value =0.002) (Figure 5B), as expected. Stratified by high and low levels of the biomarker panel expression, Kaplan-Meier plotter (kmplot.com/analysis/) was used to assess the survival outcomes of ESCC patients from databases including TCGA, EGA, and GEO etc. (12). Patients with higher biomarker expression level had significantly impaired overall survival (OS) compared with the low-expression subset [median OS, 18.9 versus 42.1 months; hazard ratio (HR), 2.29; 95% confidence interval (CI): 1.00–5.23; log-rank P=0.044] (Figure 5C).

To further test the discriminative potential of the 12 NCRT-resistant biomarkers, we performed immunofluorescence staining of the biomarkers using

formalin-fixed paraffin-embedded (FFPE) slides from an independent cohort of NCRT-treated ESCC patients. Each biomarker was stained on pre-treatment (NCRT) biopsied primary tumor samples from five pCR patients and five non-pCR patients. Biomarker positive score (BPS) was defined as the percentage of tumor cells with positive biomarker staining at any intensity (the number of positive tumor cells divided by the total number of tumor cells). The BPS values of the 12 biomarkers are shown in Figure 5D (P values for *t*-test are listed in Table S10). Ten out of the 12 biomarkers had significantly elevated expression in the tumor cells of the non-pCR patients using two-sample *t*-test, including IFIT3 (P=0.000), BST2 (P=0.000), SAMHD1 (P=0.000), HLA-B (P=0.000), EIF2AK2 (P=0.001), GBP1 (P=0.002), IFI44L (P=0.003), MX1 (P=0.006), STAT1 (P=0.03), ISG15 (P=0.04) (Figure 5E and Figure S5).

Discussion

Trimodality therapy with NCRT followed by surgery is currently the standard treatment for locally advanced esophageal cancer. However, approximately 60% of ESCCs and ~80% of esophageal adenocarcinomas cannot achieve a complete pathological response due to intrinsic or acquired tumor chemoradiation resistance. Currently, the mechanism of resistance to NCRT in esophageal cancer has not been comprehensively investigated using a systemic multiomics approach. Moreover, treatment response biomarkers evidenced by high-throughput techniques are still lacking (9).

In our study, we aimed to unveil the biological basis for therapeutic resistance by jointly analyzing genomics, transcriptomics, and proteomics data from pre-treatment biopsy samples of 24 ESCC patients. Our findings highlighted the interferon signaling pathway and its upstream RLR pathway as key players in poor responders. At the genetic level, we identified FBXW7 loss-of-function mutations and chromosome 9p21.3 copy number deletion as sensitive genetic variations to NCRT. We linked these variations to the interferon and RLR pathway expressions, validated in the TCGA ESCC dataset. Based on proteomic and transcriptomic insights, we proposed a 12-protein biomarker panel (STAT1, EIF2AK2, MX1, BST2, TRIM21, SAMHD1, IFI44L, GBP1, PARP14, ISG15, HLA-B, and IFIT3) from the ISGs family to identify treatment-resistant patients. Preliminary validation in the TCGA ESCC subset and confirmation in an independent ESCC cohort supported the potential of these biomarkers.

Constitutive activation of the IFN pathway and

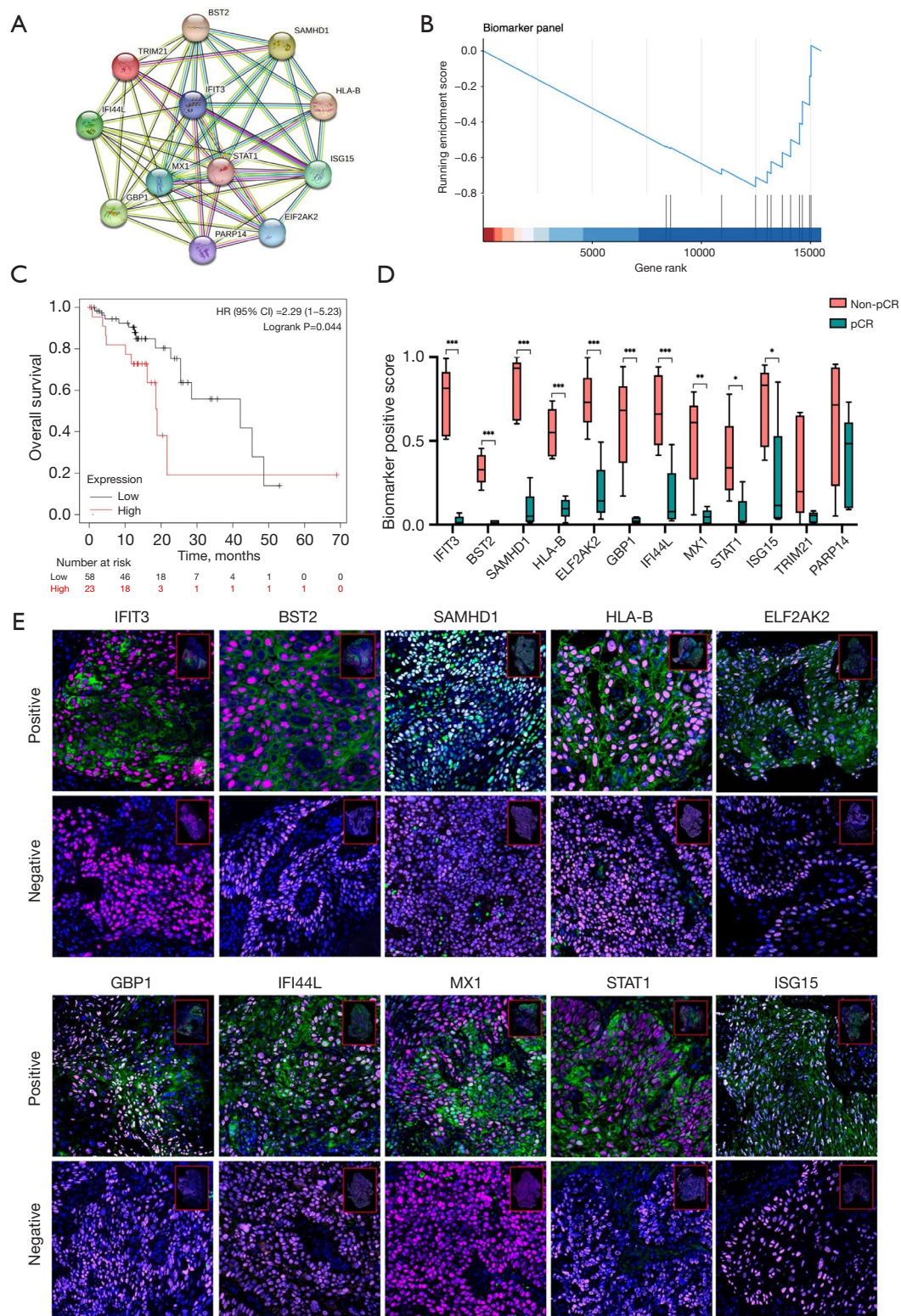


Figure 5 Type I IFN signaling pathway genes as biomarkers for NCRT treatment resistance. (A) Protein-protein interaction network of biomarker proteins plotted by STRING. Edges represent protein-protein associations. Known interactions: blue edge—from curated databases; magenta edge—experimentally determined. Predicted interactions: green edge—gene neighborhood. Others: light green

edge—text-mining; black edge—co-expression. (B) GSEA analysis of the biomarker panel in the TCGA ESCC cohort shows significant enrichment of the biomarkers in non-pCR patients. (C) Survival analysis of ESCC patients using Kaplan-Meier plotter (kmplot.com/analysis/) shows a significantly prolonged median OS of 42.1 months in patients with low expression of the biomarkers, compared with the median OS of 18.9 months in patients with high expression of the biomarkers. (D) BPS for each gene. BPS is defined as the percentage of tumor cells with positive staining at any intensity. Comparisons are made using *t*-test. IFIT3 ($P=0.000$), BST2 ($P=0.000$), SAMHD1 ($P=0.000$), HLA-B ($P=0.000$), EIF2AK2 ($P=0.001$), GBP1 ($P=0.002$), IFI44L ($P=0.003$), MX1 ($P=0.006$), STAT1 ($P=0.026$), ISG15 ($P=0.038$), TRIM21 ($P=0.073$), PARP14 ($P=0.0306$). (E) The positive and negative staining of representative samples for each biomarker gene. Blue—DAPI; pink—P63 (tumor cell staining); green—biomarker. *, $P<0.05$; **, $P<0.01$; ***, $P<0.005$. BPS, biomarker positive score; ESCC, esophageal squamous cell carcinoma; GSEA, gene set enrichment analysis; HR, hazard ratio; NCRT, neoadjuvant chemoradiotherapy; OS, overall survival; pCR, pathologic complete response; TCGA, The Cancer Genome Atlas.

expression of ISGs in cancer are related to genotoxic agents' treatment resistance (25). In a previous study, a series of STAT1-centered ISGs that were found to be highly expressed in radioresistant tumor nu61, and were designated as IFN-related DNA damage signature (IRDS). IRDS were proven to be associated with radioresistance and chemoresistance in different cell lines, including melanoma, breast cancer, lung cancer, and colon cancer (26,27). In clinical settings, a classifier incorporating seven ISGs, namely STAT1, IFI44, IFIT3, OAS1, IFIT1, G1P2, and MX1, successfully categorized patients' IRDS status. The classifier was validated across multiple independent clinical breast cancer datasets, demonstrating its reliability in predicting recurrence risk following radiotherapy and chemotherapy. Additionally, elevated expression levels of certain other ISGs, including DDX60, STAT1, OAS1, IFI6, and IFI27, were found to be associated with unfavorable radiotherapy outcomes in cancer patients (28). Our proposed NCRT treatment biomarkers demonstrated a significant alignment with those documented in the literature, affirming the reliability of our biomarker panel.

The mechanisms by which cancer cells constitutively overexpress ISGs and how elevated ISGs promote radioresistance and chemoresistance are poorly understood. Extensive literature supports that STAT1 is the core gene responsible for therapeutic resistance and oncogenic phenotypes (25). Boelens *et al.* found that exosome RNAs (exoRNA) originating from cancer stroma could induce ISGs in cancer cells through RIG-I-dependent activation of STAT1, which would then amplify the NOTCH3 signaling to expand the therapy-resistant cells with a stemness phenotype (15). Specific ISGs downstream of STAT1 were also reported to promote oncogenicity or genotoxic stress protection. For example, ISG15 was involved in reinforcing the stem-like phenotype of pancreatic ductal adenocarcinoma cancer stem cells (29). IFIT1 and IFIT3

were found to promote tumor growth and metastasis in oral squamous cell carcinoma (30). PARP14 was found to promote cell-cycle progression through cyclin D1 mRNA stabilization, and its overexpression is related to treatment resistance and poor prognosis in multiple cancers (31,32).

FBXW7, known as a tumor suppressor gene, is a key component of E3 ligase that targets the degradation of a series of proto-oncogenes, such as cyclin E and MYC. Its mutations are commonly observed in different cancer types. In our study, the mutation frequency of FBXW7 was 56% in the pCR group and 0% in the non-pCR group, and the mutation types included missense and frameshift mutations. These mutations included missense and frameshift variants, which either disrupted FBXW7 dimerization or its ability to bind to substrates. FBXW7 loss-of-function mutations have been reported to increase radiation sensitivity. Yang *et al.* demonstrated that FBXW7 knockout led to enhanced radiotherapy efficacy both *in vitro* and *in vivo* as a result of impaired double-strand break repair (33). In another research, depletion of FBXW7 was found to sensitize cancer cells to radiation through stabilization of p53 and induction of cell cycle arrest and apoptosis (19). Besides its established role in DNA damage repair, our results also suggest FBXW7 mutations confer chemoradiation sensitivity through impaired RLR and IFN signaling pathways. While early research seldom discusses FBXW7's role in immunity, a few recent studies have revealed its importance in regulating the IFN signaling pathway. Song *et al.* (34) first described the role of FBXW7 in antiviral immunity as a stabilizer of RIG-I, and FBXW7 knockout led to decreased RIG-I protein levels and impaired type I IFN signaling. In a murine melanoma model, a lack of FBXW7 was also found to be associated with diminished expression of both RIG-I and MDA-5, as well as impaired induction of IFN signaling.

In esophageal cancer, chromosome 9p21.3 deletion is the most frequent homozygous deletion among all CNAs,

and it is thought to be one of the early driving events in precancer lesions (35). Co-deletion of the IFN cluster in the 9p21.3 deletion has been associated with the disruption of type I IFN signaling, thus causing tumor immune evasion and immunotherapy resistance (20,36). Our results show that deletion of 9p21.3 sensitizes ESCC to chemoradiation, potentially through impaired IFN pathway activation.

Our study had some limitations. Genetic variations related to NCRT resistance are highly heterogeneous, and the gene mutations and CNVs proposed in this study are insufficient to explain all scenarios. Furthermore, multiomics sequencing was not performed in all the recruited samples owing to the quality control of the frozen specimens. Only 13 patients had complete sequencing data from all three omics levels. In addition, mechanistic validation experiments were performed in a limited number of ESCC cell lines, and further confirmation in additional models would strengthen the generalizability of these findings.

In summary, our multiomics analysis provided valuable insights into the distinctive biological profiles of ESCC patients responding differently to NCRT. Through the integration of genomic, transcriptomic, and proteomic data, we uncovered the consistent activation of the IFN and RLR signaling pathways as prominent characteristics in NCRT-resistant patients. Additionally, we introduced a promising ISG-based biomarker panel with the potential to predict treatment resistance in these patients.

Conclusions

In conclusion, our multiomics analysis identified constitutive activation of the type I interferon and RLR pathways as a key feature of NCRT resistance in ESCC. FBXW7 loss-of-function mutations and 9p21.3 IFN-cluster deletion were associated with chemoradiosensitivity through downregulated IFN signaling. A 12-protein ISG biomarker panel showed potential to identify treatment-resistant patients prior to therapy. These findings may inform individualized treatment strategies upon prospective validation.

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None.

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2026-1-0201/dss>

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Data Sharing Statement: Available at <https://tcr.amegroups.com/article/view/10.21037/tcr-2026-1-0201/dss>

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki and its subsequent amendments. This study was approved by the Shanghai Chest Hospital Institutional Review Board (IRB) (approval No. KS2040). The IRB determined that the requirement for informed consent was waived due to the retrospective nature of the study, in accordance with institutional and national ethical guidelines.

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