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A close-up graphic image of a tumor cell

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ORIGINAL RESEARCH ARTICLE

Identification of *BAK1* as a novel prognostic biomarker for liver cancer based on the mining of liver cancer pyroptosis-related genes

Yiyang Chen[1], Wanbang Zhou[1], Yiju Gong[1], and Xi Ou[2]*

[1]Anhui Medical University, Hefei City, Anhui Province, 230032, China

[2]Peking University Shenzhen Hospital, Futian District, Shenzhen, Guangdong, 518036, China

Abstract

Hepatocellular carcinoma is a type of digestive tract cancer that has a high incidence and a poor prognosis. Pyroptosis, a newly discovered and proven method of pro-inflammatory programmed cell death, is linked to tumor development, patient prognosis, and response to therapy. In this study, the prognostic genes of liver cancer were obtained; their expressions were extracted from The Cancer Genome Atlas database; differential analysis, survival analysis, and clinical correlation analysis were performed; and a nomogram was constructed to predict the survival rate. Based on the expression of *BAK1* gene, all samples were then divided into two groups: High expression and low expression. Enrichment analysis, immunological analysis, and drug susceptibility analysis of differential genes were subsequently performed in that order. *BAK1* expression was found to be significantly higher in liver hepatocellular carcinoma (LIHC). High *BAK1* expression levels were found to be linked to cancer development and poor prognosis. To assess the diagnostic value of *BAK1* in LIHC, a receiver operating characteristic curve was drawn. In addition, there were significant differences in drug sensitivity between high and low *BAK1* expression in 90 drugs. The *BAK1* gene may be a good potential LIHC diagnostic marker, an oncogene in the occurrence and progression of liver cancer, a new prognostic biomarker, and a potential therapeutic target for liver cancer.

Keywords: *BAK1*; Liver cancer; Pan-cancer; Immunity; Biomarkers; Prognosis

***Corresponding author:**
Xi Ou
(bdszyyox@163.com)

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1. Introduction

Hepatocellular carcinoma is a type of digestive tract cancer that has a high incidence and a poor prognosis. In recent years, the survival rate of liver cancer has improved due to the use of surgical resection as the primary treatment method and the development of immunotherapy[1]. Despite continuous advancements in medical technology, in the face of rising incidence, it is difficult to detect liver cancer in the early stages, and once it occurs, it metastasizes easily and has a poor prognosis[2]. Liver cancer is also one of the leading causes of cancer-related death, and the overall 5-year survival rate for patients with liver cancer remains <15%[3,4]. Therefore, novel diagnostic markers for liver cancer are urgently needed to improve the present therapeutic environment for liver cancer. Pyroptosis is a newly discovered and proven method of programmed cell

death with pro-inflammatory characteristics. It is classified into caspase-1-dependent classical pyroptotic pathway and caspase-4/5/11-dependent non-classical pyroptotic pathway. Pyroptosis is characterized by deoxyribonucleic acid (DNA) breakage, cell membrane rupture, and the release of pro-inflammatory proteins[5,6]. According to research, the expression of caspase-1 is low in liver cancer tissue[7]. Other research has demonstrated that hypoxia-induced caspase-1 activation and the subsequent generation of different inflammatory factors in liver cancer tissues and cell lines can promote cancer cell invasion and metastasis[8]. Pyroptosis not only impedes tumor cell proliferation but also creates a microenvironment that promotes tumor cell development[9,10]. Given the importance of pyroptosis in malignancies, the aim of this work was to identify HCC pyroptosis-related genes (PRGs) and investigate their implications in HCC prognosis.

To identify prognosis-related pyroptosis genes, the prognostic value of 52 PRGs in 115 HCC patients from the Gene Expression Omnibus (GEO, GSE 76427) cohort was examined. Following the selection of *BAK1* target gene, its expression level was obtained from The Cancer Genome Atlas (TCGA) database. In addition to the construction of a nomogram, differential analysis, survival analysis, and clinical correlation analysis were carried out to predict the survival rate. Subsequently, all samples were separated into two groups based on *BAK1* gene expression: High and low expression. Enrichment analysis, immunological analysis, and drug sensitivity analysis were performed on the differential genes. The role of *BAK1* in predicting prognosis and immunotherapy response in patients with liver cancer was investigated.

2. Materials and method

2.1. Data sources

The clinically relevant data and gene expression of liver cancer were downloaded from TCGA database (https://portal.gdc.cancer.gov/). The GEO (https://www.ncbi.nlm.nih.gov/geo/) was also used in this work. For the following analysis, a GEO HCC cohort (GSE 76427) and a TCGA cohort were collected. Thereafter, the transcriptome and clinical data were combined and ID transformed. Fifty-two pyroptosis-related genes (REACTOME PYROPTOSIS) were obtained from previously published studies and the Molecular Signatures Database (MSigDB) (http://www.broad.mit.edu/gsea/msigdb/)[11,12]. TCGA and GEO data were integrated in R studio using "limma" and "sva" packages, and the expression of the PRGs was retrieved from the merged data. Finally, the survival analysis of pyroptotic genes was performed to obtain the prognosis-related pyroptotic genes.

2.2. Gene expression and survival prognostic analysis

Using the "Diff Exp" module on the Tumor Immunity Estimation Resource (TIMER) website (http://timer.cistrome.org/) and the R studio software, we investigated *BAK1* expression in 33 human tumor and normal control tissues from the TCGA database. In LIHC, the packages "limma," "ggplot2," and "ggpubr" performed differential and pairwise differential analyses on *BAK1*. Kaplan–Meier curves created by the R studio programs "survival" and "survminer" were used to analyze the differences in survival between subtypes. Univariate and multivariate independent prognostic analyses were then carried out to determine if *BAK1* could be used independently of other prognostic indicators.

2.3. Clinical correlation analysis and coexpression analysis

Clinical correlation analyses and heatmaps were created in R studio using "limma," "ComplexHeatmap," and "ggpubr" packages. Genes that share the same promoter as *BAK1* were identified. A correlation coefficient larger than zero between the two indicates that the gene is positively regulated by *BAK1*, while a correlation coefficient lesser than zero indicates that the gene has a negative regulatory interaction with *BAK1*. The filter condition of the coefficient of correlation was corFilter = 0.6; the filter condition of the correlation test *P*-value was pFilter = 0.001, and the coexpression circle graph was drawn based on the coexpression results.

2.3. Gene enrichment analysis

The samples were separated into two groups with high and low *BAK1* expression levels, respectively, using "limma" and "pheatmap" packages in R studio. A gene heat map with differences between the high and low expression groups was generated. The logFCfilter parameter was set to 1, the fdr filter condition was fdrFilter = 0.05, and the adjusted *P*-value was 0.05. We performed Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis of differential genes in R studio program using "org.Hs.eg.db," "clusterprofiler," and "enrichplot" packages to further investigate the enrichment of probable pathways of differential genes in different groups. To further explore the enrichment of potential pathways of differential genes in different groups, we performed KEGG and GSEA enrichment analysis.

2.4. Immune correlation analysis and drug sensitivity analysis

Through differential analysis of immune cells, immune cells with statistical significance between high and low *BAK1* expression groups were discovered, and differential analysis

was performed on them. To assess the relationship between high and low *BAK1* expression groups and immune cell infiltration content, the CIBERSORT algorithm was used, and the filter condition for immune cell infiltration findings was set to pFilter = 0.05. Immune checkpoint correlation analysis was then performed to investigate the relationship between immune checkpoint genes and *BAK1*, with the filter condition of correlation test *P*-value set to pFilter = 0.001. The immunoscores of liver cancer patients were gathered from http://tcia.at/, and immunotherapy analysis was performed in R studio program using "limma" and "ggpubr" packages. The immunohistochemical pictures of BAK1 protein in various cancer and normal tissues were obtained from http://www.proteinatlas.org/(Human Protein Atlas [HPA] database). We estimated the half maximal inhibitory concentrations (IC50s) of compounds collected from the Genomics of Drug Sensitivity in Cancer (GDSC) website in the TCGA project of the LIHC dataset to identify possible medications for clinical application in LIHC therapy. The IC50 of the chemical, acquired from the GDSC website, in LIHC patients was predicted using R studio's "pRRophetic" package, with the filter condition for *P*-value set to pFilter = 0.001.

2.5. Building a nomogram scoring system

The clinical variables and risk scores were integrated to construct a predictive nomogram in R studio using "rms," "survival," and "regplot" packages. Each clinical feature and risk rating in the nomogram was assigned a score; the total score was calculated by summing the scores for all clinical features and risk ratings in each sample. The accuracy of anticipated 1-, 3-, and 5-year survival events was described using calibration plots.

2.6. Statistical analysis

R version 4 was used for all statistical studies. Statistical significance was defined as $P < 0.05$.

3. Results

3.1. Expression and survival analysis of BAK1

Table 1 lists the 33 cancers studied in this work. The HCC cohort (GSE76427) used has 115 patients in total. We analyzed the PRGs in 115 HCC patients using R studio packages "survival" and "limma." $P < 0.05$ was deemed statistically significant for prognosis. Figure 1A depicts the gene coexpression relationship, revealing that *GSDME*, *CHMP4B*, *CHMP3*, *BAK1*, and *NOD2* are high-risk genes that are strongly associated with prognosis. In view of the limited number of research on the prognosis and immunity of *BAK1* expression in liver cancer, we chose *BAK1* and used TIMER 2.0 database to investigate *BAK1* expression in pan-cancer. Figure 1B shows that the expression of *BAK1* was significantly upregulated in 11 cancer types (bladder urothelial carcinoma [BLCA], breast invasive carcinoma [BRCA], cholangiocarcinoma [CHOL], esophageal carcinoma [ESCA], glioblastoma multiforme [GBM], head-and-neck squamous cell carcinoma [HNSC], liver hepatocellular carcinoma [LIHC], lung adenocarcinoma [LUAD], lung squamous cell carcinoma [LUSC], stomach adenocarcinoma [STAD], and uterine corpus endometrial carcinoma [UCEC]; all $P < 0.001$), whereas it was

Table 1. 33 cancer types used in this study

Abbreviation	Full name
ACC	Adrenocortical carcinoma
BLCA	Bladder urothelial carcinoma
BRCA	Breast invasive carcinoma
CESC	Cervical squamous cell carcinoma and endocervical adenocarcinoma
CHOL	Cholangiocarcinoma
COAD	Colon adenocarcinoma
DLBC	Lymphoid neoplasm diffuse large B-cell
ESCA	Esophageal carcinoma
GBM	Glioblastoma multiforme
HNSC	Head and neck squamous cell carcinoma
KICH	Kidney chromophobe
KIRC	Kidney renal clear cell carcinoma
KIRP	Kidney renal papillary cell carcinoma
LAML	Acute myeloid leukemia
LGG	Low grade glioma
LIHC	Liver hepatocellular carcinoma
LUAD	Lung adenocarcinoma
LUSC	Lung squamous cell carcinoma
MESO	Mesothelioma
OV	Ovarian serous cystadenocarcinoma
PAAD	Pancreatic adenocarcinoma
PCPG	Pheochromocytoma and paraganglioma
PRAD	Prostate adenocarcinoma
PEAD	Rectum adenocarcinoma
SARC	Sarcoma
SKCM	Skin cutaneous melanoma
STAD	Stomach adenocarcinoma
TGCT	Testicular germ cell tumors
THCA	Thyroid carcinoma
THYM	Thymoma
UCEC	Uterine corpus endometrial carcinoma
UCS	Uterine carcinosarcoma
UVM	Uveal melanoma

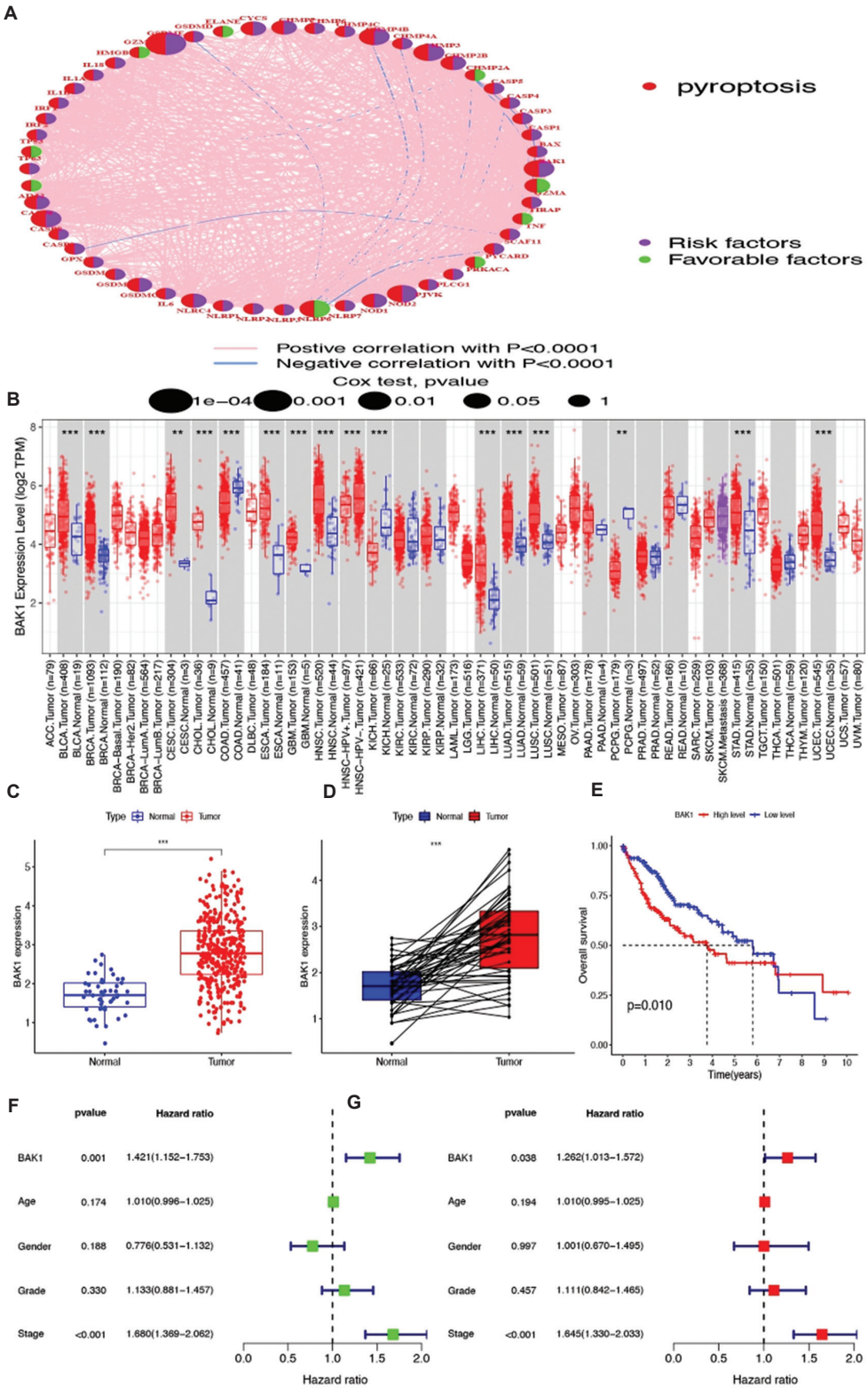

Figure 1. (A) Pyroptosis-related genes interactions in liver cancer. (B) *BAK1* expression levels in several cancer types and matching normal tissues. (C) *BAK1* differential analysis in liver hepatocellular carcinoma (LIHC). (D) Pair-wise differential analysis of *BAK1* in LIHC. (E) *BAK1* survival analyses in LIHC. (F–G) Univariate and multivariate independent prognostic study of *BAK1*. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

significantly downregulated in two additional cancer types (colon adenocarcinoma [COAD] and kidney chromophobe [KICH]; all $P < 0.001$). We then looked at how *BAK1* differed between HCC and non-HCC tissues. Figures 1C and D reveal that *BAK1* was expressed at a greater level in HCC tissues and that this level differs considerably from that of non-HCC tissues. We subsequently compared the prognosis of HCC patients with high and low *BAK1* expression; the results revealed that the overall survival rate of HCC patients with high *BAK1* expression was considerably lower than that of patients with low *BAK1* expression (Figure 1E). According to univariate and multivariate independent prognostic analyses (Figures 1F and G), *BAK1* is associated with prognosis and can be a prognostic factor independent of other factors. In univariate Cox regression analysis, the hazard ratio (HR) and 95% confidence interval (CI) were 1.421 and 1.152 – 1.753, respectively ($P = 0.001$); in multivariate Cox regression analysis, the HR was 1.262, and the 95% CI was 1.013 – 1.572 ($P = 0.038$).

3.2. Relationship between BAK1 expression and clinical features

A clinical correlation study was performed to determine if *BAK1* expression differed between clinical groups. *BAK1* exhibited significant differences in gender, tumor grade, tumor stage, and T stage. Figure 2A shows that BAK1 expression does not differ significantly with age. Figure 2B demonstrates that BAK1 expression is significantly higher in female HCC patients than in males. Figure 2C depicts *BAK1* expression levels in various tumor stages, with substantial statistical differences between Stage 1 and Stage 2, as well as Stage 1 and Stage 3. Figure 2D shows a statistically significant difference in BAK1 expression among T1, T2, and T3, but no such difference was seen in the other T stages. The clinical correlation heatmap in Figure 2E shows that the three clinical parameters tumor grade, tumor stage, and T stage have substantial statistical differences between high and low *BAK1* expression groups. Coexpression analysis revealed that *BAK1* is positively regulated by *SMARCD1*, *MFSD10*, *RCC2*, *CDK16*, *PKM*, and *MACROH2A1* but negatively regulated by *GLYATL1*, *ALDH2*, *CDO1*, *DCXR*, and *SLC27A5* (Figure 2F). The diagnostic value of *BAK1* in LIHC was assessed by drawing a receiver operating characteristic (ROC) curve (Figure 2G). We discovered that the area under the ROC curve was 0.694, 0.582, and 0.611 at 1, 3, and 5 years, indicating that this gene may be a good prospective LIHC diagnostic marker.

3.3. Analysis of differential genes and construction of nomogram

All samples were classified into high and low expression groups according to the expression of the target gene *BAK1*, and genes with differences in expression between the high and low expression groups were identified (Figure 3A). The differential genes were found to be involved in neuroactive ligand-receptor interaction, cytokine-cytokine receptor interaction, adenosine 3',5'-cyclic monophosphate (cAMP) signaling pathway, and hematopoietic cell lineage by KEGG enrichment analysis (Figure 3B). We then ran a GSEA, and the results in Figure 3C showed that the four functions HUMORAL IMMUNE RESPONSE MEDIATED BY CIRCULATING IMMUNC, IMMUNOGLOBULIN COMPLEX, IMMUNOGLOBULIN COMPLEX CIRCULATING, and IMMUNOGLOBULIN RECEPTOR BINDING were active in the high BAK1 expression group, and STEROID HYDROXYLASE ACTIVITY is active in the high and low expression group of BAK1. The five pathways FATTY ACID METABOLISM, GLYCINE SERINE AND THREONINE METABOLISM, PEROXISOME, PRIMARY BILE ACID BIOSYNTHESIS, and RETINOL METABOLISM were all active in the low *BAK1* expression group, as shown in Figure 3D. We constructed a nomogram using risk classes and clinical data to predict the 1-, 3-, and 5-year survival in LIHC patients, as shown in Figure 3E. Assuming a patient's composite score is 394, the 1-year survival rate is 0.941, the 3-year survival rate is 0.882, and the 5-year survival rate is 0.839. Correlation plots revealed that the observed and expected rates of survival in LIHC patients at 1, 3, and 5 years were in perfect agreement (Figure 3F).

3.4. Expression of BAK1 and immunity and drug sensitivity

Figure 4A shows a differential study of immune cells, which revealed a statistically significant difference in dendritic cell activation between high and low BAK1 expression groups, suggesting that the activation is favorably regulated by BAK1. Figure 4B, we then examined the relationship between BAK1 and immunological checkpoint-related genes. BAK1 positively regulates CD276, CD86, CD80, TNFRSF8, TNFSF15, LGALS9, TNFRSF18, PDCD1, VTCN1, and HAVCR2, while IDO2 and BAK1 have a skewed relationship. Unfortunately, no significant statistical difference was observed when receiving CTLA-4 and PD-1 treatment regardless of whether BAK1 expression was high or low (Figure 4C and D). Figure 4E and F illustrate the immunohistochemistry (IHC) status of BAK1 in normal and cancerous liver tissue, respectively. Representative IHC photos reveal that BAK1 protein is more abundant in tumors than in non-tumor tissues. We then identified drugs that showed substantial changes in their sensitivity in patients with high and low BAK1 expression. Ninety drugs were discovered. Figure 4G to Figure 4N, Fluorouracil, bosutinib, bleomycin, cyclopamine, and other drugs were

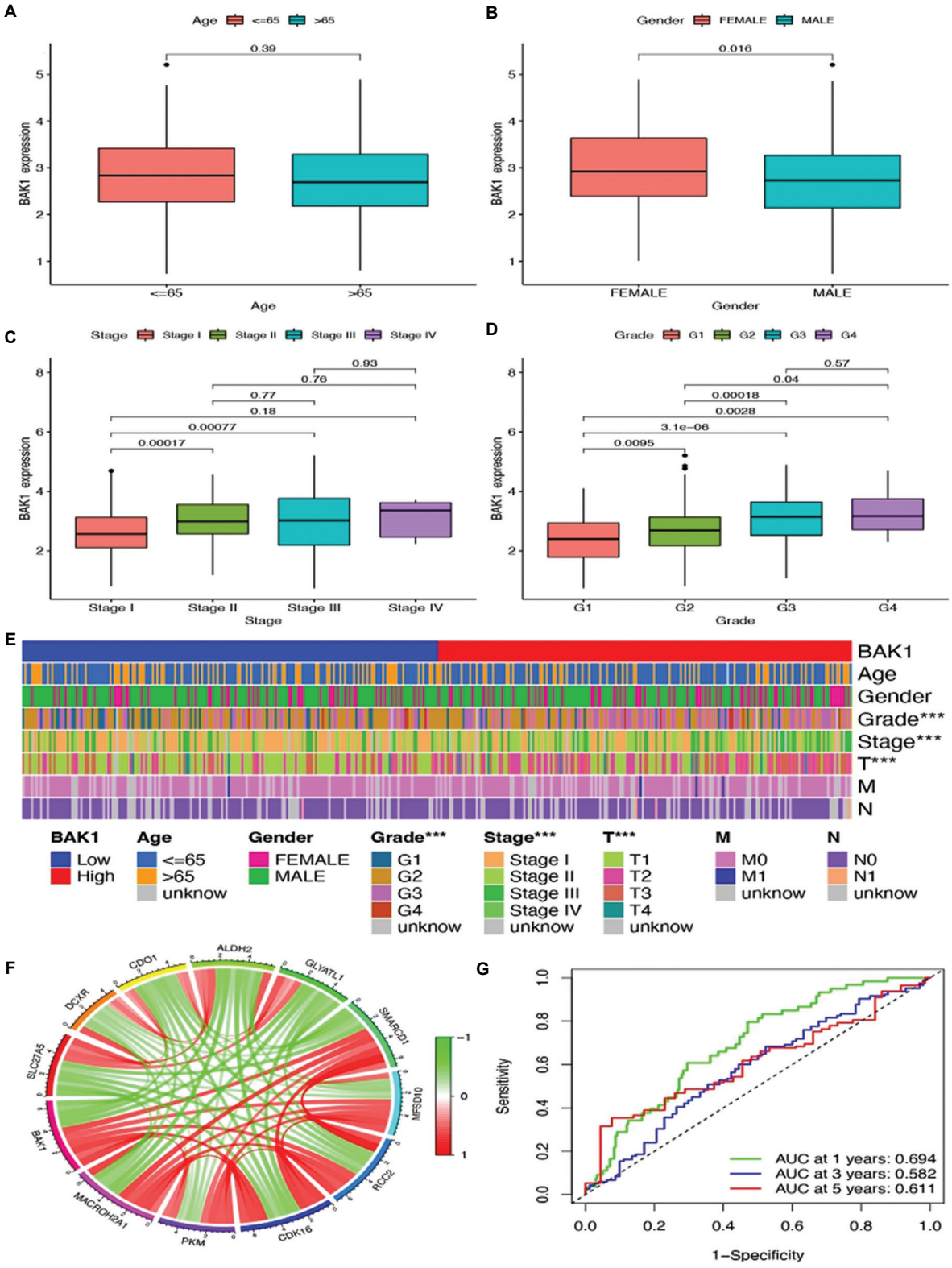

Figure 2. (A–D) *BAK1* correlation analysis by age, gender, tumor grade, and tumor stage. (E) Heatmap of *BAK1* and clinical feature correlations. (F) Genes with *BAK1* coexpression relationship. (G) Receiver operating characteristic (ROC) curves used in GSE 76427 to predict 1-, 3-, and 5-year ROC curves.

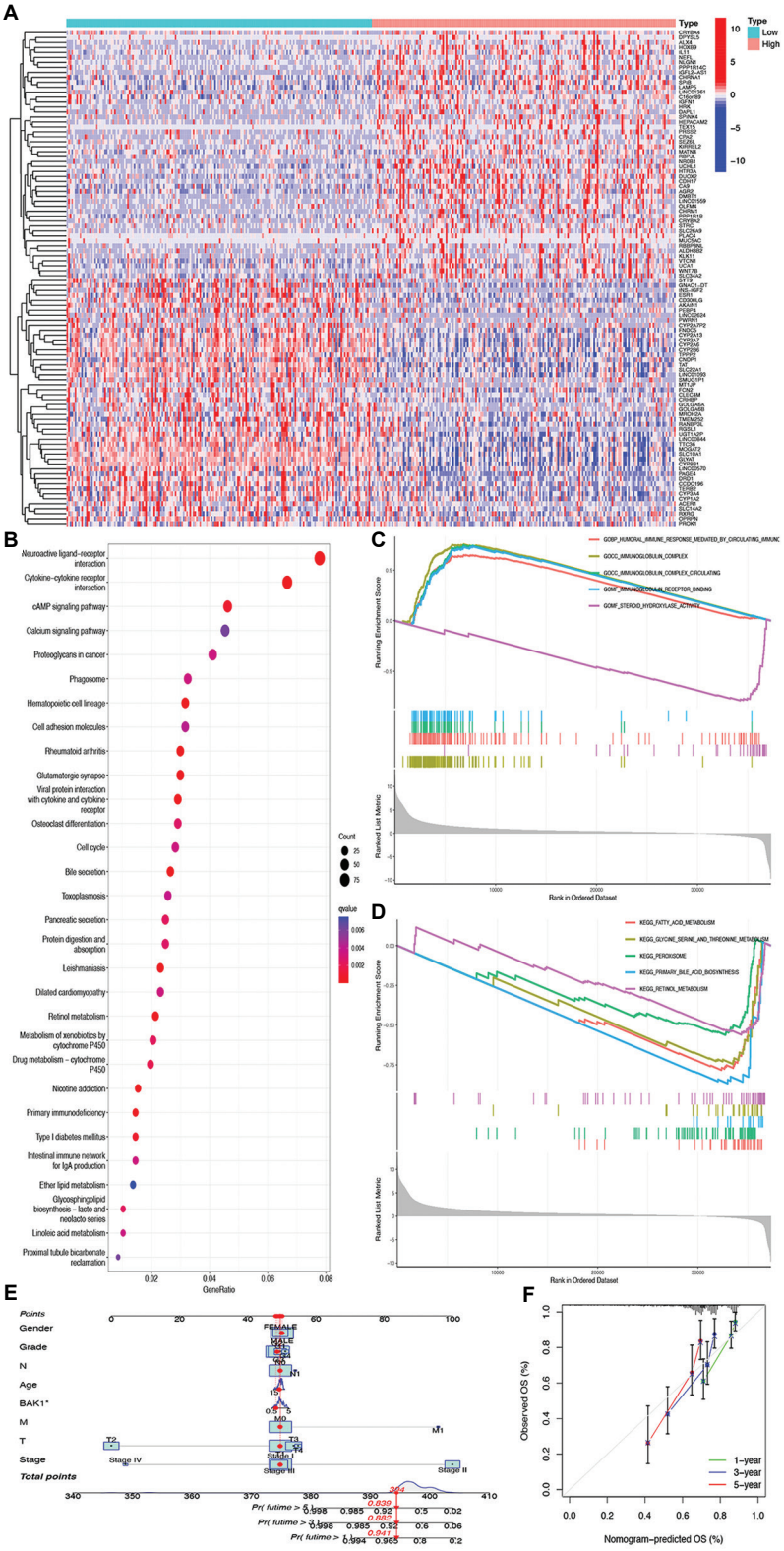

Figure 3. (A) Differential gene analysis in groups with high and low *BAK1* expression. (B) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of differential genes. (C and D) Gene Set Enrichment Analysis (GSEA) of differential genes. (E) A nomogram to predict the 1-, 3-, and 5-year survival in LIHC patients. (F) Calibration curves for the nomogram used in GSE 76427 to predict the 1-, 3-, and 5-year survival.

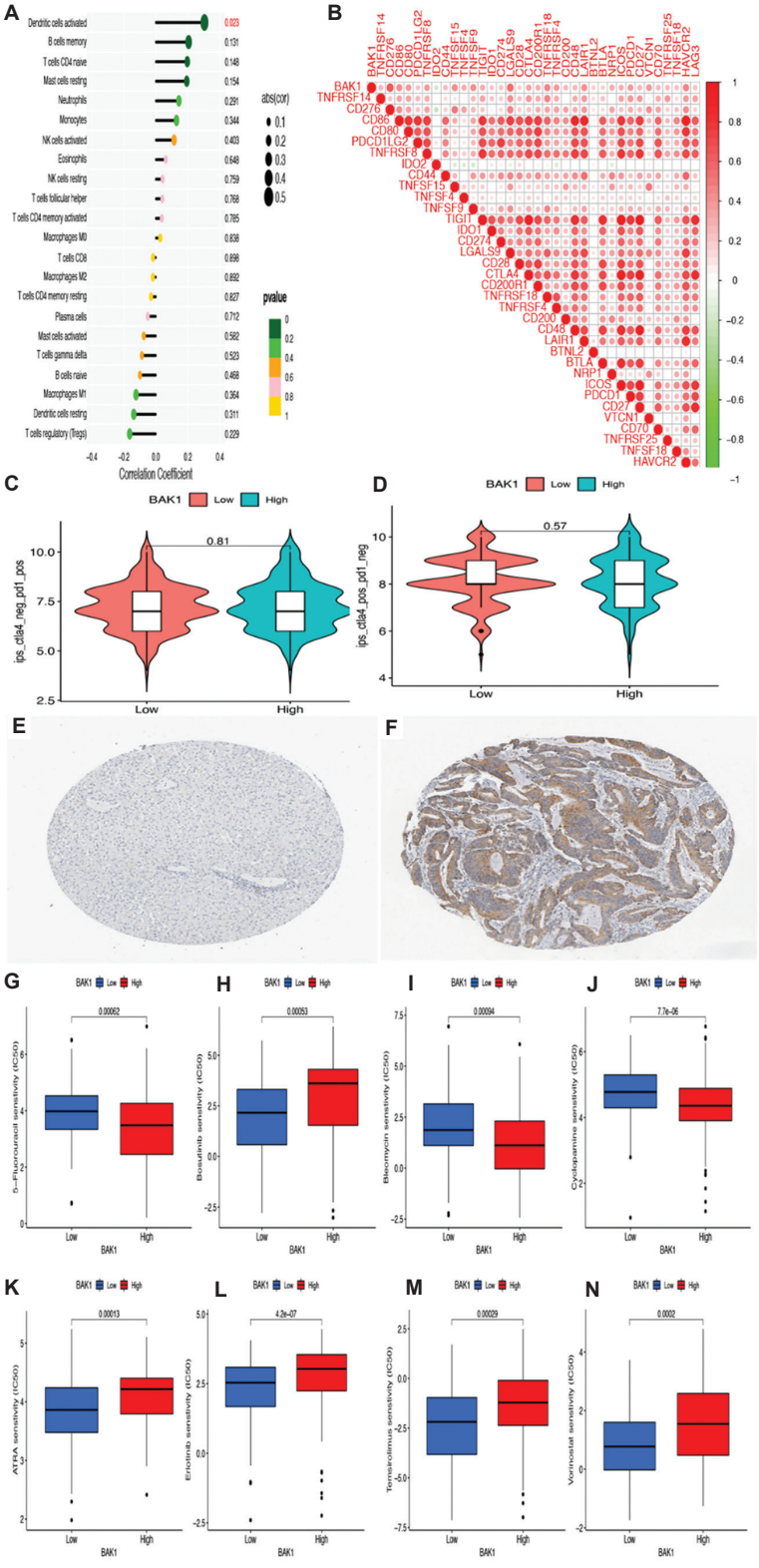

Figure 4. (A) *BAK1* and immune cell correlation diagram. (B) *BAK1* association with immune checkpoint-related genes. (C and D) *BAK1* and immunotherapy correlation study. (E and F) Immunohistochemical pictures of *BAK1* protein in normal and malignant liver tissues. (G–N) Drugs with significant differences in drug sensitivity in high and low *BAK1* expression.

found to be more sensitive to BAK1 expression in patients. Patients with reduced BAK1 expression are more sensitive to all-trans retinoic acid (ATRA), erlotinib, temsirolimus, vorinostat, and other drugs.

4. Discussion

One of the most prevalent primary malignant tumors worldwide is HCC. HCC is often diagnosed at an advanced stage in the majority of patients due to the disease's gradual onset; the early diagnosis of HCC poses a challenge. Certain physiological activities can alter the changes to its indicators due to the poor sensitivity and specificity of early screening, such as serum alpha-fetoprotein (AFP)[13]. Therefore, identifying molecular biomarkers that fully reflect the biological characteristics of liver cancer is a critical link in the early diagnosis and treatment of patients with liver cancer[14,15]. Previous research has looked at the role of pyroptosis-related genes in anti-tumor activity[16]; thus, we set out to investigate the role of pyroptosis-related genes in the prognosis of patients with liver cancer. By examining the predictive value of PRG in 115 HCC patients in the HCC cohort (GSE76427), we discovered that *GSDME*, *CHMP4B*, *CHMP3*, *BAK1*, and *NOD2* are all high-risk genes, which are closely associated with the prognosis of patients. We chose *BAK1* as the target gene for this investigation due to the lack of prior research on the prognosis and immunity of *BAK1* expression in liver cancer. The *BAK1* (BCL2-antagonist/killer1) gene is found on the outer mitochondrial model and belongs to the B-cell lymphoma/leukemia-2 (*BCL2*) gene family[17,18]. The previous research has found *BAK1* to be a prognostic biomarker in women with lung adenocarcinoma[19] as well as a prognostic marker in individuals with colon cancer[20]. It reduces apoptosis and enhances cisplatin resistance in non-small cell lung cancer when combined with cancer-associated fibroblast (CAF)-derived exosomal miR-103a-3p[21]. It has also been shown that *BAK1* is a tumor suppressor gene that regulates cell cycle through microRNA in patients with endometrial cancer[22]. However, there are several studies that have linked BAK1 to HCC diagnosis and prognosis.

The expression of *BAK1* in pan-cancer was investigated using the TIMER database. The expression of *BAK1* was shown to be highly upregulated in 11 cancer types (*i.e.*, BLCA, BRCA, CHOL, ESCA, GBM, HNSC, LIHC, LUAD, LUSC, STAD, and UCEC), but significantly downregulated in two cancer types (i.e., COAD and KICH). We then looked at the differences in *BAK1* expression between HCC and non-HCC tissues; we discovered that *BAK1* was expressed at a greater level in HCC tissues than in non-HCC tissues. The predictive analysis of high and low *BAK1* expression groups revealed that patients with high *BAK1* expression had a considerably poorer overall survival rate than patients with low *BAK1* expression. According to univariate and multivariate independent prognostic analyses, *BAK1* is associated with prognosis and can be independent of other factors. These findings show that *BAK1* could become one of the diagnostic indicators for HCC prognosis. We also constructed a nomogram containing risk classes and clinical characteristics to predict the 1-, 3-, and 5-year survival in LIHC patients, thus making practical application easier. Assuming a patient's comprehensive score is 394, the 1-year survival rate is 0.941, the 3-year survival rate is 0.882, and the 5-year survival rate is 0.839, indicating that it has good prediction ability. The ROC curve was used to assess the diagnostic value of *BAK1* in LIHC, and the area under the ROC curve was 0.694, 0.582, and 0.611 at 1, 3, and 5 years, respectively. According to the findings, the *BAK1* gene may be a good potential LIHC diagnostic marker. Furthermore, we discovered a link between clinical phase features and *BAK1* expression. The findings revealed that *BAK1* expression differed significantly by gender, tumor grade, tumor stage, and T stage, with *BAK1* being more evident in female patients. *BAK1* expression increased with tumor grade in females, and there were significant statistical differences between the other groups except for the expression between G3 and G4. There were statistically significant variations in *BAK1* expression between Stage 1 and Stage 2, as well as Stage 1 and Stage 3. In addition, there was a statistically significant difference in *BAK1* expression among T1, T2, and T3. According to coexpression study, *BAK1* was found to be positively regulated by *SMARCD1*, *MFSD10*, *RCC2*, *CDK16*, *PKM*, and *MACROH2A1*, but negatively regulated by *GLYATL1*, *ALDH2*, *CDO1*, *DCXR*, and *SLC27A5*. The finding of these genes may bring new ideas for multi-biomarker diagnosis in the future.

The differential study of immune cells showed that the difference between dendritic cells activated in groups with high and low *BAK1* expression was statistically significant and exhibited a positive regulatory interaction with *BAK1*. The immune checkpoint-related gene *HAVCR2* is upregulated, while *IDO2* and *BAK1* are downregulated. The finding of these immune checkpoint-related genes may provide specific ideas and directions for the later use of *BAK1* in liver cancer immunological research. Subsequently, we identified drugs that showed substantial changes in sensitivity with high and low *BAK1* expression, of which 90 drugs were discovered. Fluorouracil, bosutinib, bleomycin, cyclopamine, and other drugs were found to be more sensitive to *BAK1* expression. Patients with reduced *BAK1* expression are more sensitive to ATRA, erlotinib, temsirolimus, and other drugs. The discovery of these medications may provide more alternatives for the treatment

of liver cancer in the future, as well as new directions for clinical use and drug development. Our current study, inevitably, has certain limitations. First, our work is based on RNA-sequencing data, derived from public dataset (TCGA), and lacks clinical trials; thus, the predictive ability of all pyroptosis-related prognostic indicators and nomograms needs to be further studied in multicenter clinical trials and prospective investigations. Furthermore, the prognostic role of BAK1 in hepatocellular carcinoma also needs to be further investigated, as does the underlying mechanism.

5. Conclusion

Our data demonstrate that *BAK1* expression is greatly enhanced in LIHC and that high levels of *BAK1* expression are associated with cancer progression and poor prognosis. These findings suggest that *BAK1* may be an oncogene for HCC pathogenesis and progression, as well as a novel prognostic biomarker and potential therapeutic target for HCC.

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Conflict of interest

The authors declare that there are no conflicts of interest.

Author contributions

Conceptualization: Yiyang Chen and Xi Ou
Formal analysis: Yiyang Chen and Yiju Gong
Writing – original draft: Yiyang Chen and Wanbang Zhou
Writing – review & editing: Yiyang Chen

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Peking University Shenzhen Hospital.

Consent for publication

Not applicable.

Availability of data

The datasets presented in this study can be found in online repositories. The names of the repository and accession number(s) can be found in the article.

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ACCSCIENCE PUBLISHING

Tumor Discovery

ORIGINAL RESEARCH ARTICLE

N6-methyladenosine-related long noncoding RNA is a potential biomarker for predicting pancreatic cancer prognosis

Yiyang Chen[1,2], Wanbang Zhou[2], Yiju Gong[2], and Xi Ou[2]*

[1]Anhui Medical University

[2]Peking University Shenzhen Hospital Clinical School, Futian District, Shenzhen, Guangdong Province, China

Abstract

Pancreatic cancer is a common malignant tumor of the digestive system, with insidious onset, difficult early diagnosis, easy metastasis, and poor prognosis. N6-methyladenosine (m6A) and long non-coding RNA (lncRNA) play important roles in the prognostic value and immunotherapy response of pancreatic adenocarcinoma (PAAD). Therefore, it is crucial to recognize m6A-related-lncRNAs in PAAD patients. In this study, m6A-related lncRNAs were obtained by coexpression analysis. Univariate, the Least Absolute Shrinkage, and Selection Operator (LASSO) and multivariate Cox regression analyses were performed to construct m6A-related lncRNA prognostic models. Kaplan–Meier analysis, principal component analysis, feature-rich annotation, and nomogram were used to analyze the accuracy of risk models. Potential drugs targeting this model are also discussed. A prognostic model based on m6A-related lncRNAs was constructed, potential drugs targeting this m6A-related lncRNAs feature were discovered, and the relationship with immunotherapy response was studied. Finally, a nomogram was established to predict survival in PAAD patients. This m6A-based lncRNAs risk prognostic model may be promising for clinical prediction of prognosis and immunotherapy response in PAAD patients.

Keywords: Bioinformatics; N6-methyladenosine; Long non-coding RNA; Pancreatic adenocarcinoma

***Corresponding author:**
Xi Ou
(bdszyyox@163.com)

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1. Introduction

Pancreatic adenocarcinoma (PAAD) is a common malignant tumor of the digestive system, with a high degree of malignancy and strong invasiveness[1,2]. Despite the continuous development of multidisciplinary comprehensive treatment, pancreatic cancer is often found at an advanced stage, and the prognosis of patients is still poor, with a median survival time of 5 – 8 months. Its onset is insidious, its early diagnosis is difficult, and it is prone to metastasis. Despite the continuous new progress in the field of comprehensive treatment of pancreatic cancer, there is still little effect in improving the prognosis of patients, and the 5-year survival rate is still <9%[3,4]. Therefore, it is very important to find more effective clinical indicators for the diagnosis and treatment of pancreatic cancer patients.

N6-methyladenosine (m6A) is a dynamic methylation modification located at the N6 site of adenosine, which is the most common internal modification in eukaryotic mRNA, mediating mRNA splicing, structural switching, transport, and translation, degradation and other metabolic processes[5-7]. The disordered regulation of m6A methylation modification may affect the processing, degradation, and translation of mRNA, leading to the activation of oncogenes and the inactivation of tumor suppressor genes, which are closely related to the occurrence, development, and drug resistance of malignant tumors. M6a methylation modification involves the action of various modifying enzymes, which are the main factors regulating carcinogenesis and tumor progression[8]. Long non-coding RNA (lncRNA) is a general term for a class of non-coding RNAs longer than 200 nucleotides, which has almost no protein-coding function due to the lack of complete open reading frames. Promotion or inhibition of cancer development can affect the diagnosis and the treatment of tumors[9,10]. Changes in RNA can affect a variety of biological processes. Therefore, the role of m6A-regulated lncRNAs may be crucial for the proliferation and migration of cancer cells[11]. Besides, studies have reported that lncRNAs can promote pancreatic cancer cell proliferation and inhibition of apoptosis[12].

The m6A methylation modification process is reversible and involves a variety of enzymes (adenosine methyltransferases, demethylases, and RNA-binding proteins). Knockout of *METTL3* gene expression reduces mRNA m6A methylation modification and attenuates cancer cell proliferation, invasion, and migration[13]. The demethylase *ALKBH5* is one of the important predictors of overall survival in pancreatic cancer, and studies have found that silencing *ALKBH5* can significantly increase the proliferation, migration, and invasion of pancreatic cancer cells *in vitro* and *in vivo*, while its overexpression does the opposite[14]. The result of another study reported that the expression level of lncRNAs *KCNK15-AS1* and *ALKBH5* in pancreatic cancer tissues was significantly lower than those in normal tissues and after overexpression of *ALKBH5* in different cell lines, the KCNK15-AS1 expression was subsequently increased, while the epithelial-mesenchymal transition in pancreatic cancer cells was inhibited[15,16]. The specific role of m6A regulators in lncRNAs remains unclear. Therefore, understanding the mechanism of m6A-related-lncRNA in the development of PAAD may provide new ideas for the prognosis and treatment of pancreatic cancer patients.

In this study, the expression profiles of 14,056 lncRNAs and 23 m6A genes were extracted from the Cancer Genome Atlas (TCGA) dataset. M6A-related lncRNAs were identified using the limma package and BiocManager package in R studio software. A prognostic model was constructed based on m6A-related lncRNAs, which was then used to predict the overall survival of PAAD patients. Next, potential drugs targeting m6A-related lncRNAs were identified using publicly available drug sensitivity databases. At the same time, the relationship with immunotherapy response was explored. Finally, a nomogram was built to predict survival in PAAD patients.

2. Materials and methods

2.1. Data sources

RNA-seq transcriptome data of PAAD patients were obtained from the TCGA (https://cancergenome.nih.gov/) database and ID-transformed transcriptome data. Relevant clinical information was downloaded, and clinical information of 185 patients was extracted. The mutation data were downloaded and organized. Pancreatic cancer patients with no survival and incomplete data were excluded to avoid statistical error in this study.

2.2. Selection of m6A genes and m6A-related lncRNAs

Transcriptome expression matrix was obtained by extracting transcriptome data. MRNA and lncRNA were distinguished, and the expression levels of m6A-related genes were extracted. According to the previous studies, the expression matrix of 23 m6A genes was retrieved from TCGA[17] which includes writers (*METTL3*, *METTL14*, *METTL16*, *WTAP*, *VIRMA*, *ZC3H13*, *RBM15*, and *RBM15B*), readers (*YTHDC1*, *YTHDC2*, *YTHDF1*, *YTHDF2*, *YTHDF3*, *HNRNPC*, *FMR1*, *LRPPRC*, *HNRNPA2B1*, *IGFBP1*, *IGFBP2*, *IGFBP3*, and *RBMX*), and erasers (*FTO* and *ALKBH5*) expression data. Using the limma package and BiocManager package in R studio software (standard: corFilter > 0.4, p value Filter < 0.001), the lncRNAs related to m6A were screened, and 288 lncRNAs with coexpression relationship with m6A were identified. LncRNAs related to m6A were screened out with limma, tidyverse, ggplot2, and ggExtra packages in R studio software.

2.3. Construction and validation of prognostic models

The entire TCGA dataset was randomized into training and testing groups. A prognostic model was constructed using the training set, and the established model was validated. Subgroups including low-risk and high-risk groups were also subsequently established based on the median risk score. Combined with the survival information of PAAD

patients in TCGA, we screened the m6A-related lncRNAs involved in model construction from 288 m6A-related lncRNAs in the TCGA dataset ($P < 0.05$). This study used univariate Cox regression and the Least Absolute Shrinkage and Selection Operator (LASSO). Cox regression was performed using the R package glmnet to find m6A-related lncRNAs significantly associated with PAAD patient survival in the TCGA dataset. Multivariate Cox regression was used to analyze m6A-related lncRNAs, and finally, a m6A-related lncRNAs risk model was established. The formula for calculating the risk score is as follows:

Risk score = m6A-related lncRNAs1 × coef + m6A-related lncRNAs2 × coef +… + m6A-related lncRNAsn×coef

Where coef represents the coefficient, which is the coefficient between lncRNAs and survival. Risk curves for high and low risk were constructed using the pheatmap package in R studio software. ROC curves were constructed using the survival, survminer, and timeROC packages in R studio software. Then, model validation was performed on clinical subgroups to find out which clinical subgroups our model was applicable to.

2.4. Differential gene identification, functional analysis, and tumor mutational burden

Differentially expressed genes in high-risk and low-risk groups were identified and Gene Ontology (GO) functional analysis was performed on them. The filtering criteria of high-risk and low-risk differential genes were logFCfilter > 1 and fdrFilter < 0.05. GO functional analysis was performed using the cluster profiler package in the R studio software. The analysis threshold was determined by p-value, with $P < 0.05$ indicating significant enrichment

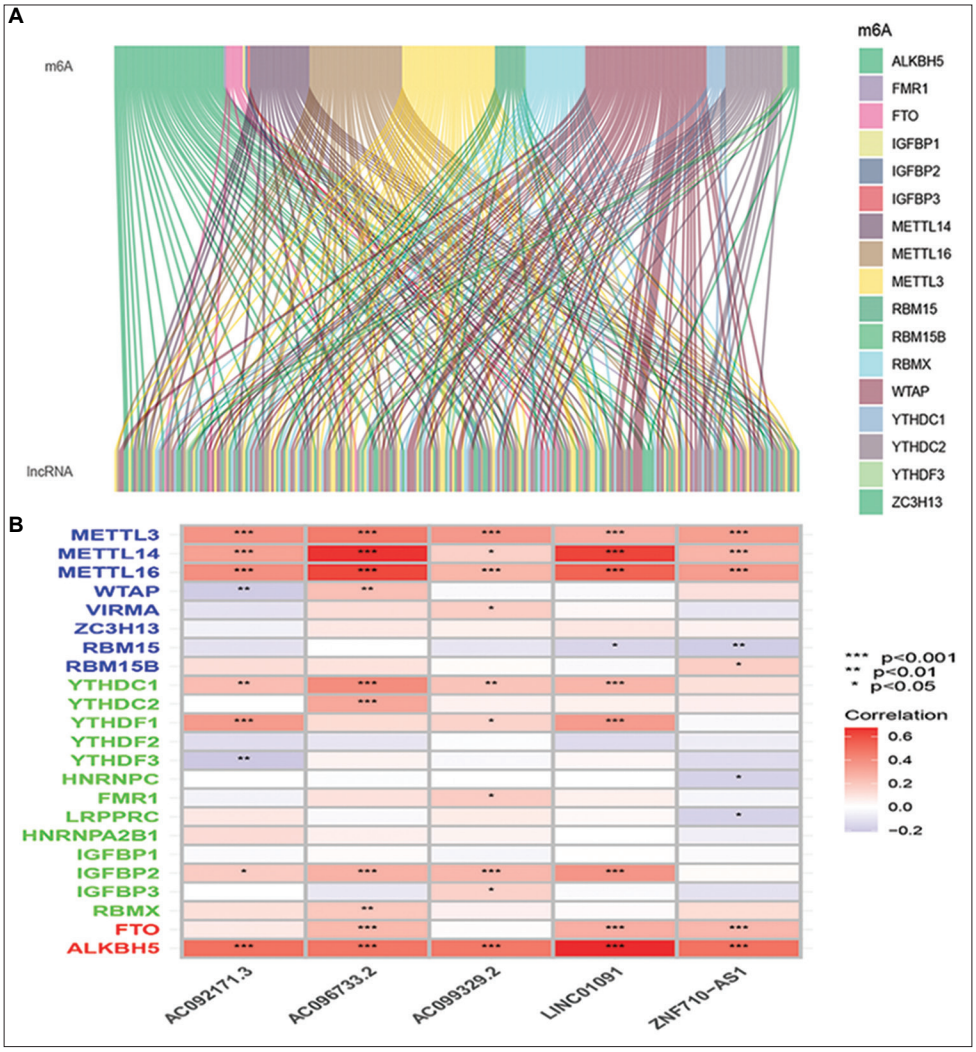

Figure 1. (A) Sankey relationship diagram of m6A genes and m6A-related lncRNA. (B) heat map of correlations between m6A genes and five prognostic m6A-related lncRNA.

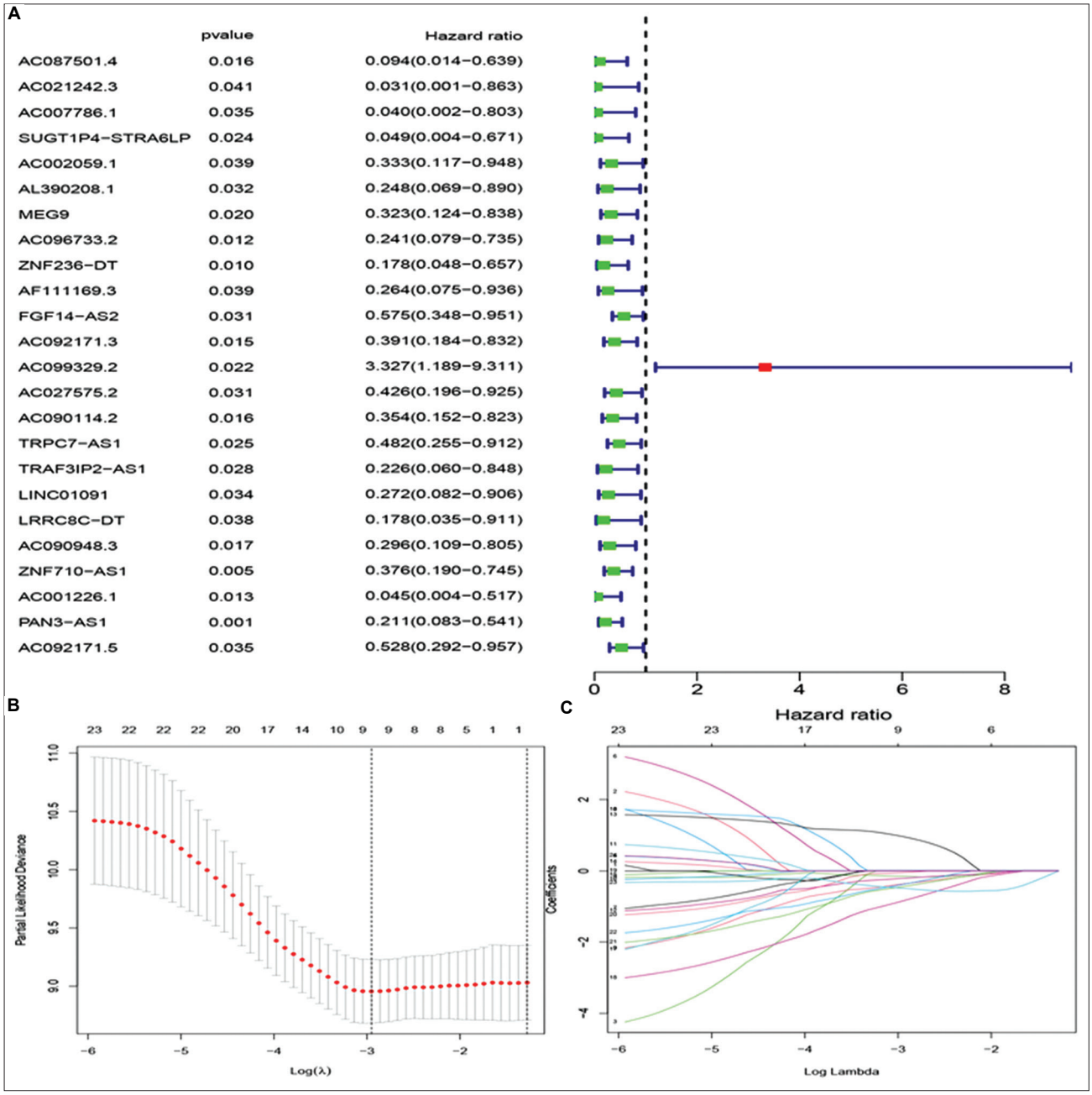

Figure 2. (A) Univariate Cox regression analysis showed that selected lncRNA was significantly associated with clinical prognosis. (B) Adjusted parameters (logλ) of OS-related proteins were selected to cross-validate error curves. A vertical imaginary line was drawn at the optimal value according to the minimum criterion and the 1-se criterion. (C) Distribution of LASSO coefficients and vertical imaginary lines for OS-associated lncRNA are plotted with values selected by ten-fold cross-validation.

of functional reviews. The ggpubr package and the limma package in R studio software were also used to analyze, whether the tumor mutation burden of the high-risk and low-risk groups was different. The survival package and survminer package in the R studio software were then used to analyze the survival of the high and low tumor mutation burden groups.

2.5. Model estimation of tumor immune microenvironment, principal component analysis, and Kaplan–Meier survival analysis

Differential immune function in high-risk and low-risk groups was screened by limma package, GSVA package, and GSEABase package in R studio software. The maftools

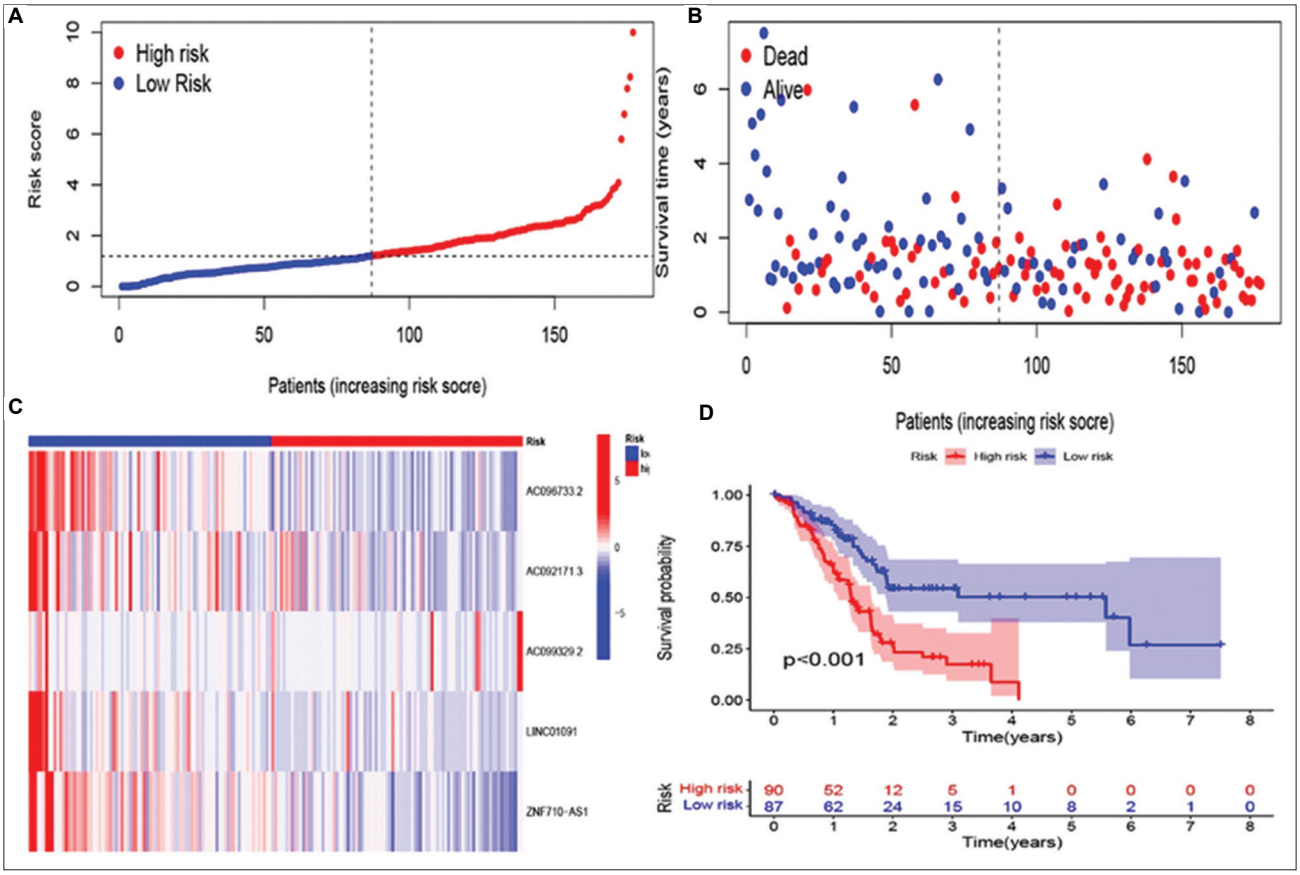

Figure 3. (A) Risk score distribution based on m6A-related lncRNAs prognostic model. (B) Different survival status and survival time of high-risk and low-risk groups. (C) Cluster analysis heatmap showing the expression criteria of 5 prognosis-related lncRNAs for each patient. (D) Kaplan–Meier survival curves of patients in high-risk and low-risk groups.

package in the R studio software was used to assess the mutation frequencies of the high-risk and low-risk groups in the model. Principal component analysis (PCA) which is used for efficient dimensionality reduction, model identification, and grouping of high-dimensional data of whole gene expression profiles, m6A genes, m6A-related lncRNAs, and risk models based on gene expression patterns visualization was performed. Kaplan–Meier survival analysis was then used to assess the diversity of survival between high- and low-risk groups. The R packages survminer and survival are the tools used for this research.

2.6. Analysis of prognostic models and screening of potential drugs

Multivariate and univariate Cox regression analyses were performed to test whether the prognostic model was an independent variable considering other clinical characteristics of PAAD patients (sex, age, tumor grade, and tumor stage). Analyses of immune evasion and immunotherapy were also performed to find out whether there were differences between high- and low-risk groups when receiving immunotherapy. The half maximal inhibitory concentration (IC50) of compounds obtained from the GDSC website Genomics of Drug Sensitivity in Cancer (https://www.cancerrxgene.org/) in the TCGA project of the PAAD dataset were calculated to obtain potential drugs for clinical use in PAAD treatment. IC50s of compounds obtained from the GDSC website were predicted in PAAD patients using the pRRophetic package in R studio software.

2.7. Construction and validation of the nomogram

The predictive power of nomogram and other predictors (age, gender, risk score, TNM stage, T stage, N stage, and M stage) for 1-, 3-, and 5-year survival was set. A calibration curve based on the Nomogram-predicted test was applied to illustrate the agreement between actual and model-predicted results.

3. Results

3.1. Identification of m6A-associated lncRNAs in PAAD patients

The matrix expression of 23 m6A genes and 14,056 lncRNAs was extracted from the TCGA database. Two hundred and

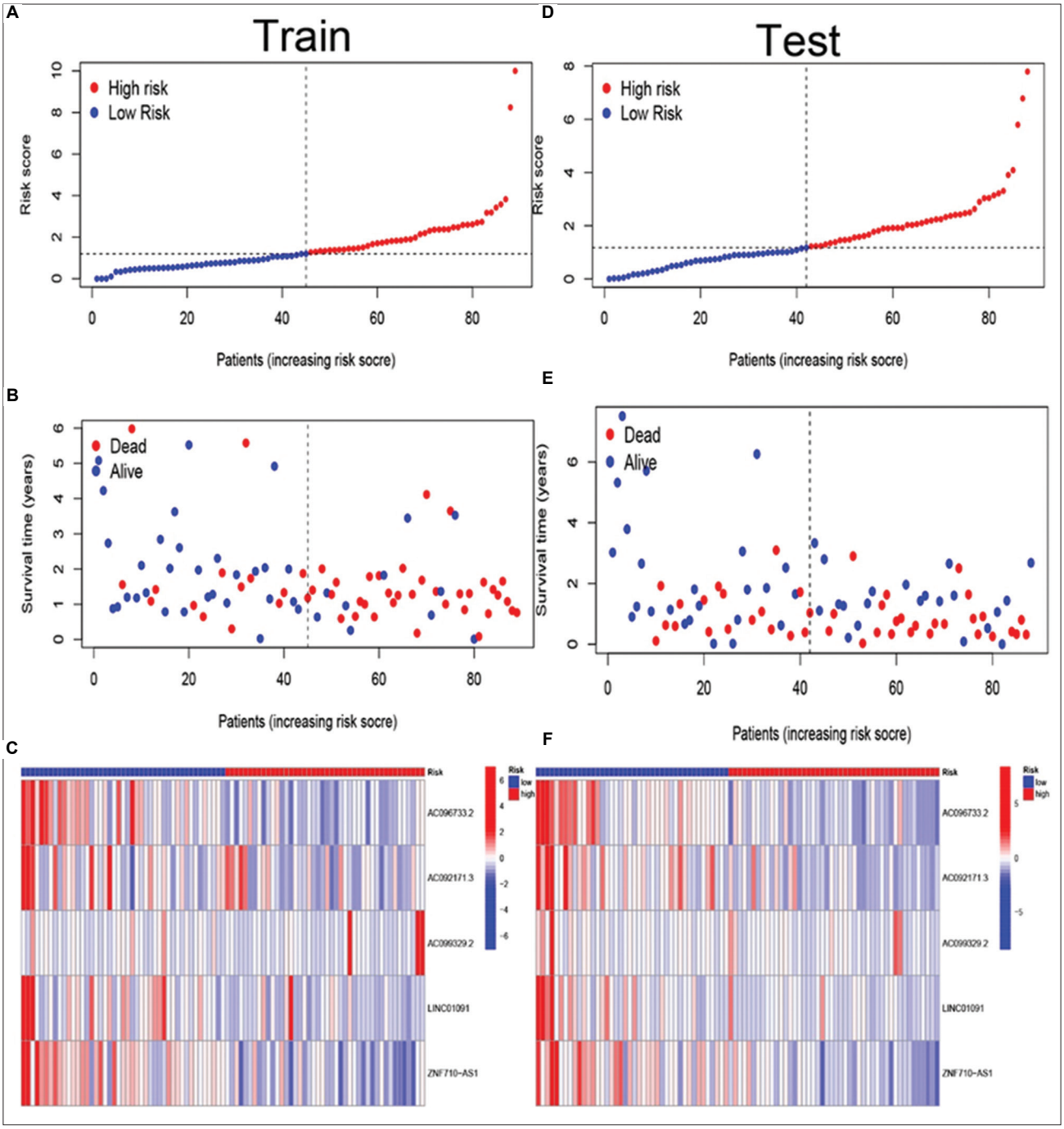

Figure 4. (A) The distribution of risk scores for the training group based on the m6A-related lncRNAs model. (B) Survival time and survival status between high-risk and low-risk groups in the training group. (C) Cluster analysis heatmap showing the displayed levels of 5 prognostic lncRNAs for each patient in the training group. (D) Distribution of risk scores for the test group based on the m6A-related lncRNAs model. (E) Survival time and survival status of high-risk and low-risk groups in the test group. (F) Cluster analysis heatmap showing the displayed levels of 5 prognostic lncRNAs for each patient in the test group.

eighty-eight lncRNAs with a coexpression relationship with m6A were identified. M6A-associated lncRNAs were defined as lncRNAs significantly associated with ≥ 1 of the 23 m6A genes (|Pearson R| > 0.4 and $P < 0.001$). In Figure 1A, a Sankey diagram of m6A-related lncRNAs is shown. In Figure 1B, a heatmap of the correlations between 23 m6A genes and 5 m6A-related lncRNAs involved in model construction, with positive correlations in red and negative correlations in blue.

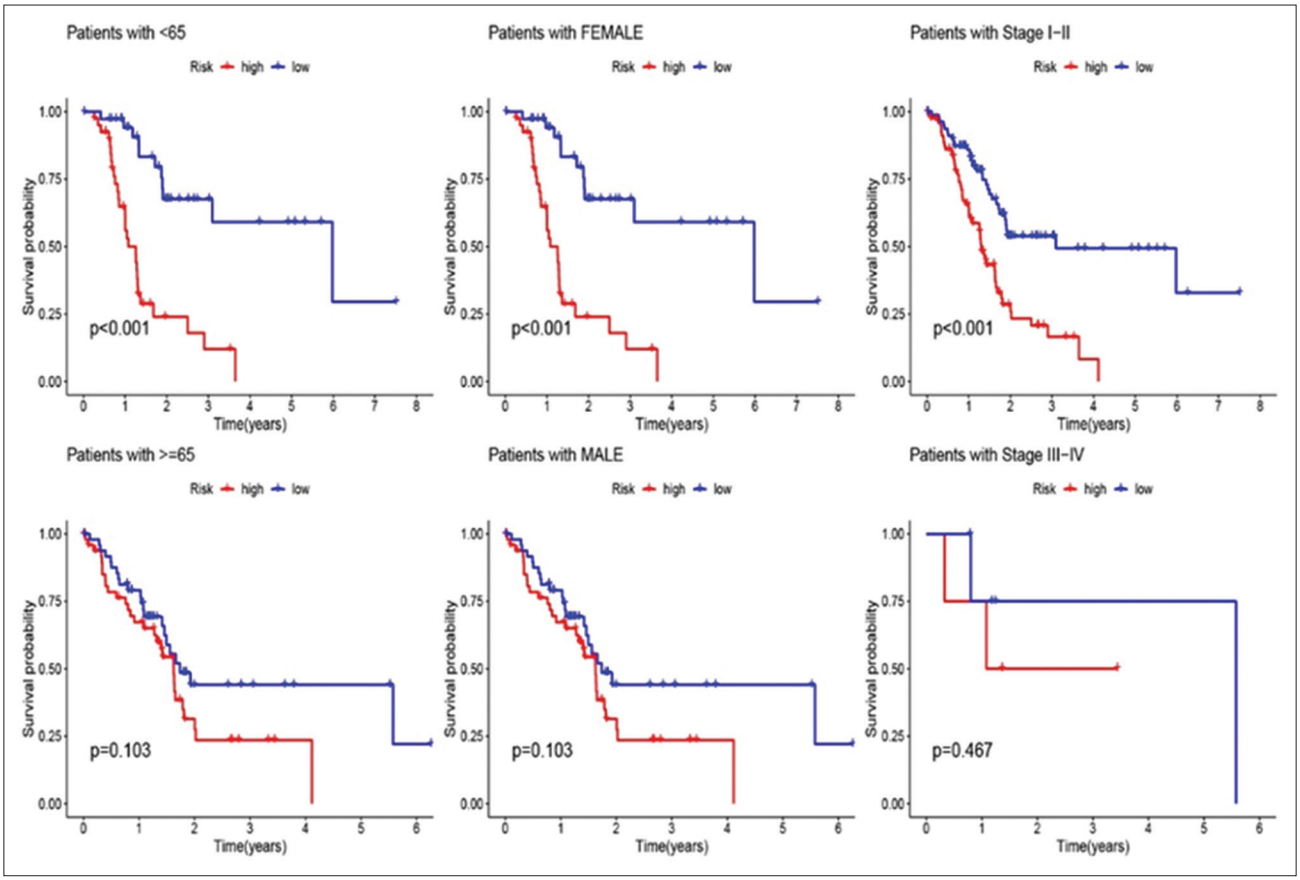

Figure 5. Kaplan–Meier curves for differences in survival between high-risk and low-risk groups by sex, age, and tumor grade.

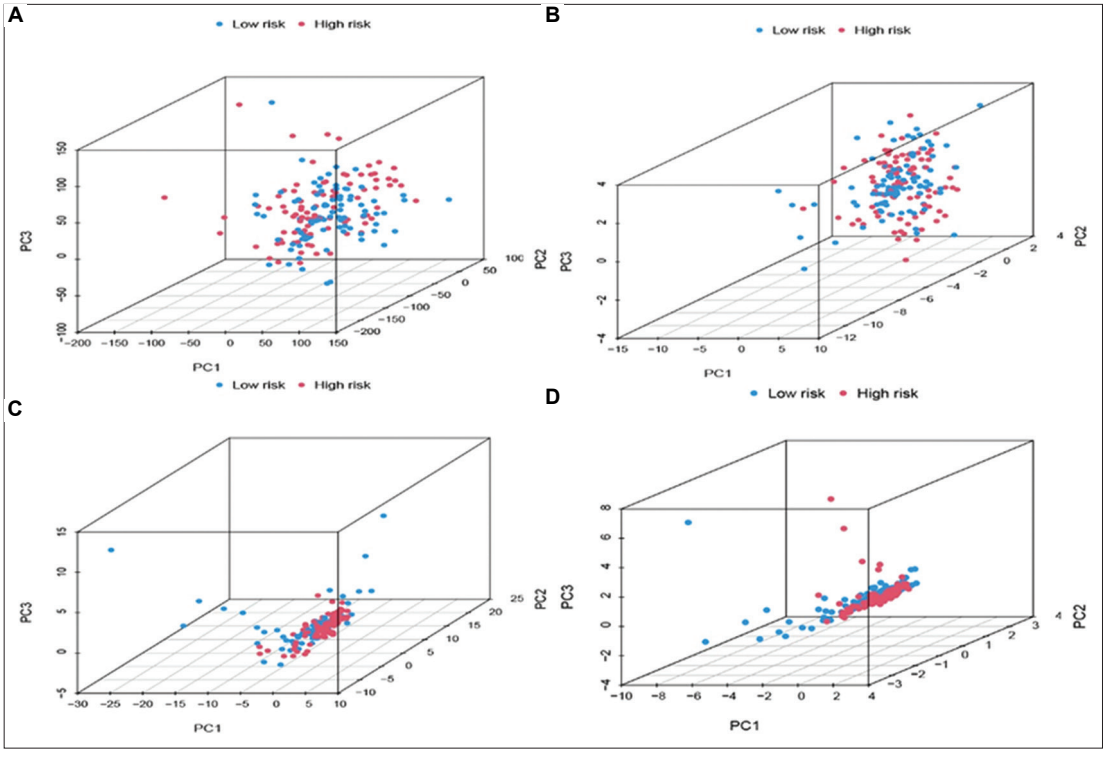

Figure 6. (A) Whole gene expression profile; (B) m6A gene; (C) m6A-associated lncRNAs; and (D) model lncRNAs.

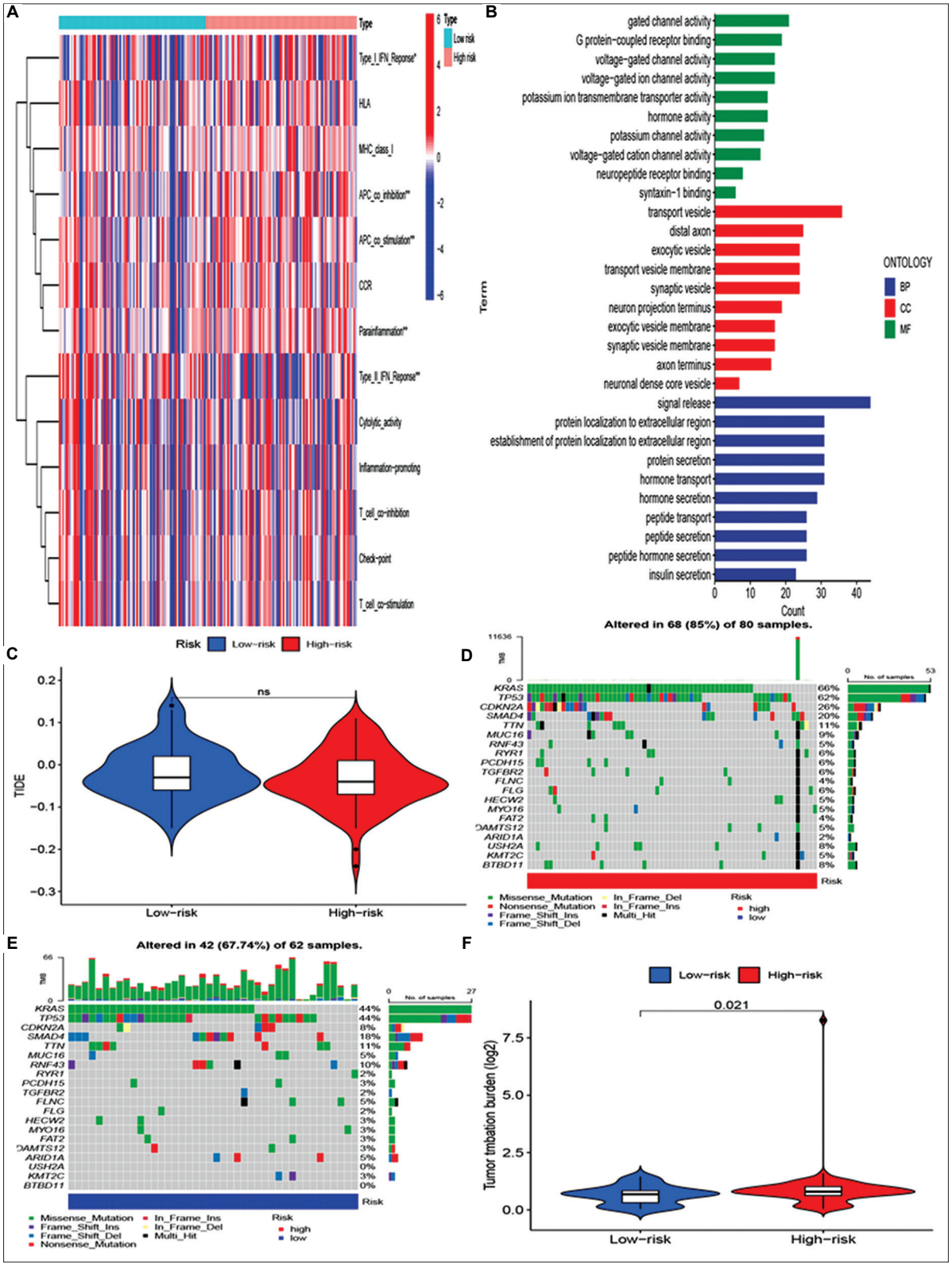

Figure 7. (A) Assignment criteria for the immune index for each PAAD patient. (B) GO enrichment analysis. (C) TIDE predictions for high-risk and low-risk patients. (D and E) Waterfall plots showing mutation information for genes with high mutation frequencies in the high-risk group (D) and the low-risk group (E). (F) Differences in TMB in high- and low-risk patients.

3.2. Construction and validation of risk models based on m6A-related lncRNAs in PAAD patients

The m6A-related prognostic lncRNAs were screened from 288 m6A-related lncRNAs in the TCGA training set using univariate Cox regression analysis. In Figure 2A, 24 m6A-related lncRNAs in the TCGA dataset were significantly associated with survival. LASSO-penalized Cox analysis is a common method for multiple regression analysis. The application of this method not only improves the prediction accuracy and interpretability of statistical models but also enables variable selection and regularization to be performed simultaneously, which can effectively identify the most available predictive markers and generate prognostic

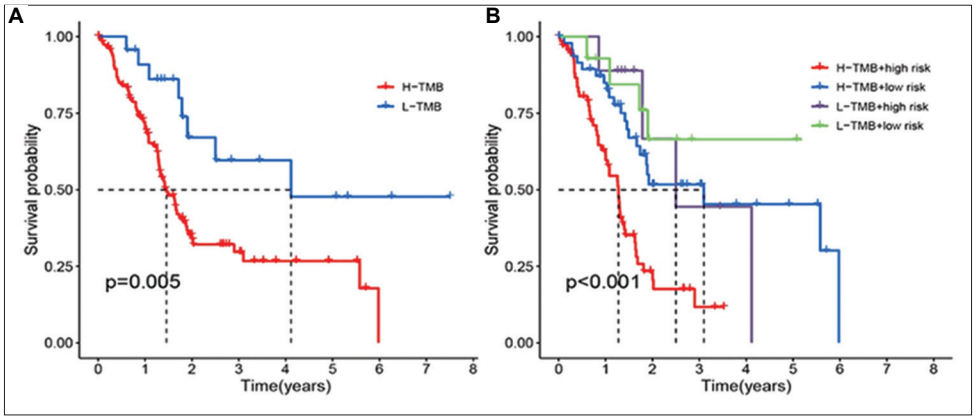

Figure 8. (A) Survival comparison of high and low tumor mutational burden. (B) Tumor mutational burden combined with patient risk score for survival analysis

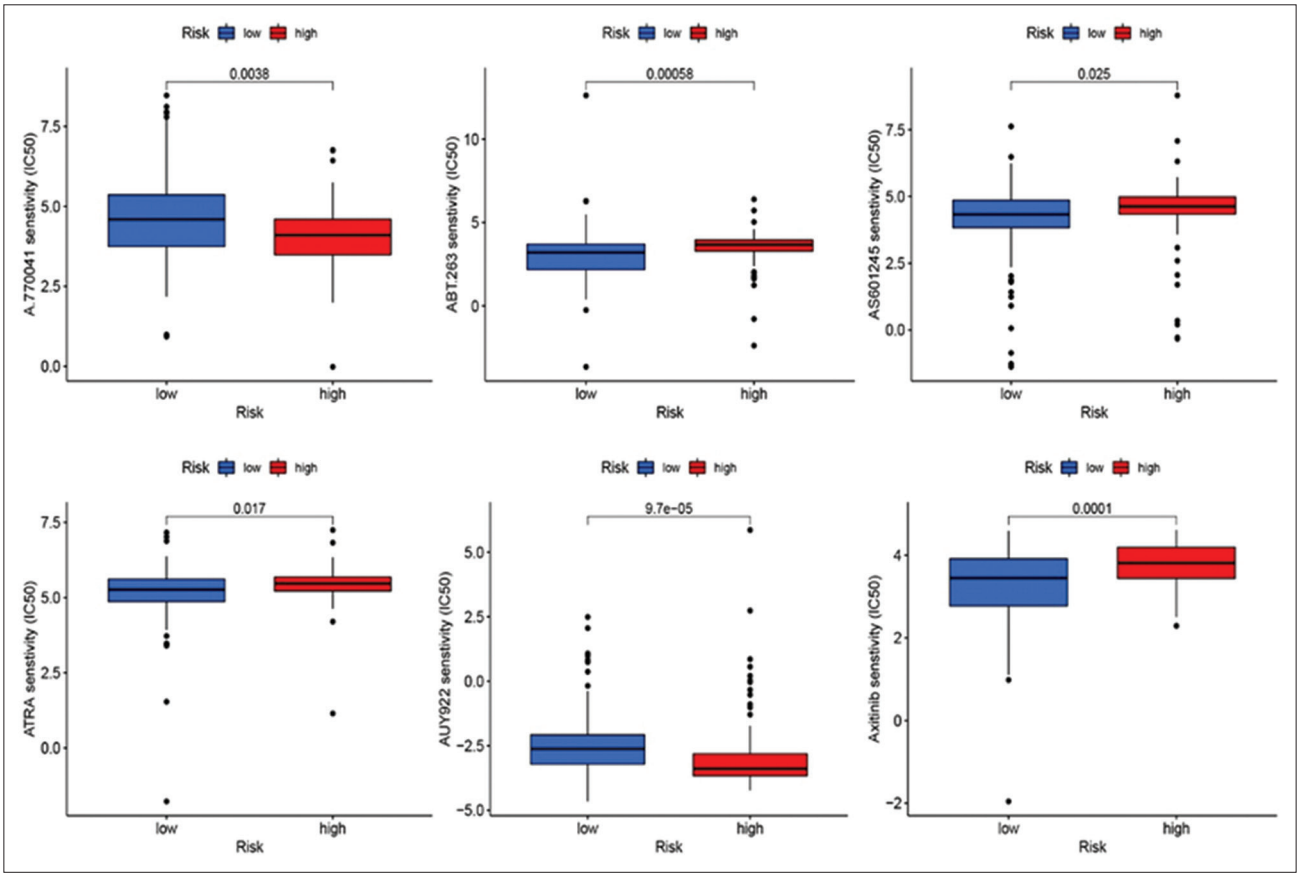

Figure 9. Six potential drugs for further analysis of PAAD patients

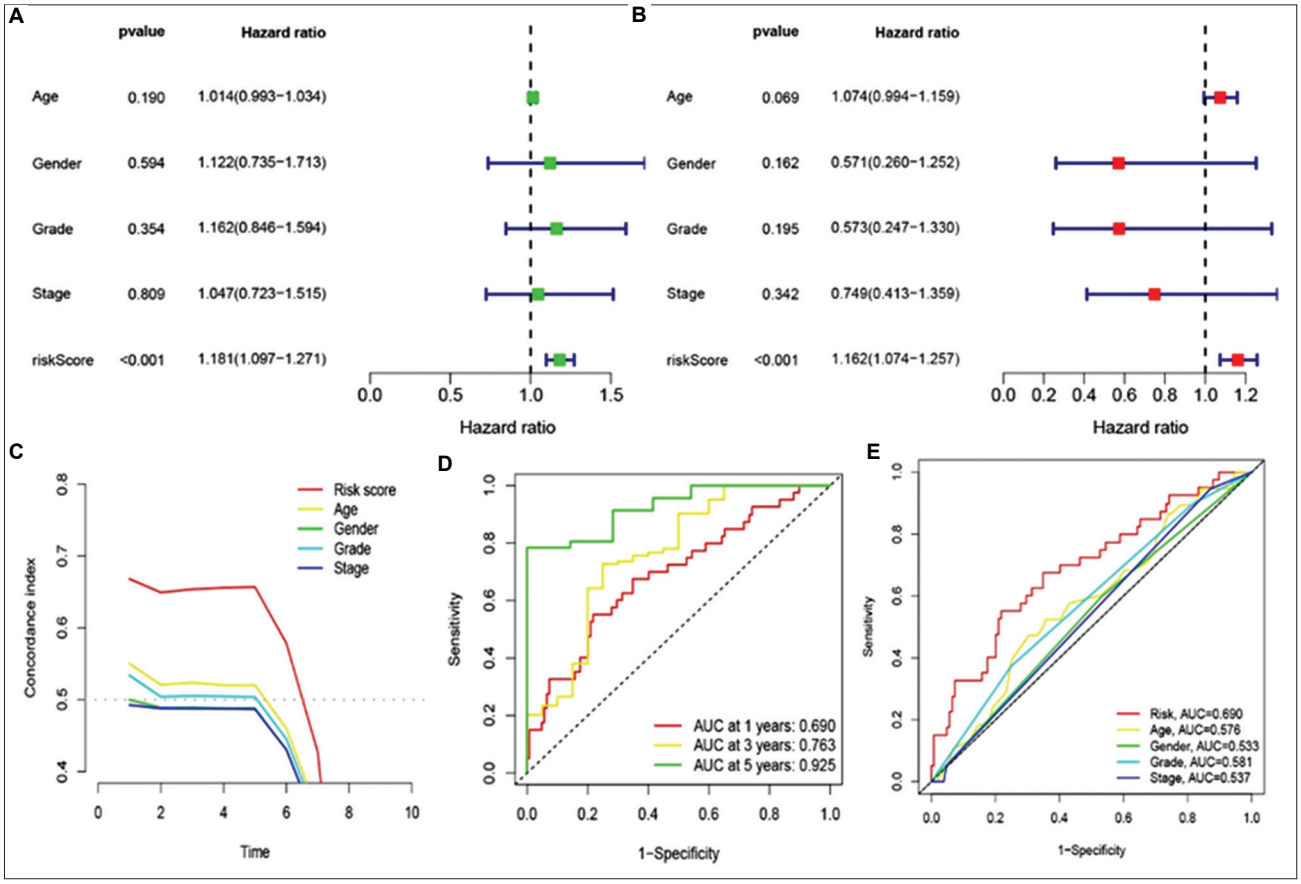

Figure 10. (A, B) Univariate and multivariate analysis of clinical characteristics and risk scores for survival. (C) Index of concordance between risk scores and clinical characteristics. (D, E) ROC curves of clinical features and risk scores.

indicators to predict clinical outcomes. The vertical-dashed line illustrates the first level value of logλ with the smallest segmentation error. Therefore, 9 m6A-related lncRNAs were selected for subsequent multivariate analysis. Next, multivariate Cox ratio hazard regression analysis was performed to distinguish autologous prognostic proteins. 5 m6A-related lncRNAs, which were prognostic proteins independently associated with survival in the training set, were used to construct risk models to assess prognostic risk in PAAD patients (Figure 2B and C). PAAD patients were divided into low-risk and high-risk groups according to the median prognostic risk grade. Figure 3A shows the distribution of risk levels for the entire set; Figure 3B shows survival status and survival time; Figure 3C shows m6A-related lncRNAs; in Figure 3D, we performed a Kaplan–Meier survival analysis, which showed that the low-risk group survived longer than the high-risk group ($P < 0.001$).

To test the prognostic power of this established model, the risk score for each patient in the training group and in the test group was calculated using a unified formula. Figure 4 depicts risk scores, survival status patterns, and risk heatmaps (Figures 4A-C for the training group; Figures 4D-F for the test group), with increasing risk levels from the left to right. Subsequently, model validation of clinical groupings was performed, as shown in Figure 5, to verify whether patients with different clinical characteristics were suitable for the model constructed in this study. The training group and test group were, further, divided into low-risk subgroup and high-risk subgroup based on age, sex, and tumor stage. The low-risk subgroup showed significantly higher survival rate than the high-risk subgroup.

3.3. Further validation of the prognostic model through principal component analysis

PCA analysis was performed in this study to test whether 23 m6A genes, 5 m6A-related lncRNAs, and model lncRNAs could have different distributions in high- and low-risk groups based on the whole gene expression profile. Figures 6A-C show that the distributions of high-risk and low-risk groups are relatively dispersed, while Figure 6D based on the model we constructed shows that the high- and low-risk groups have different distributions, indicating that the model can distinguish between high- and low-risk groups of patients.

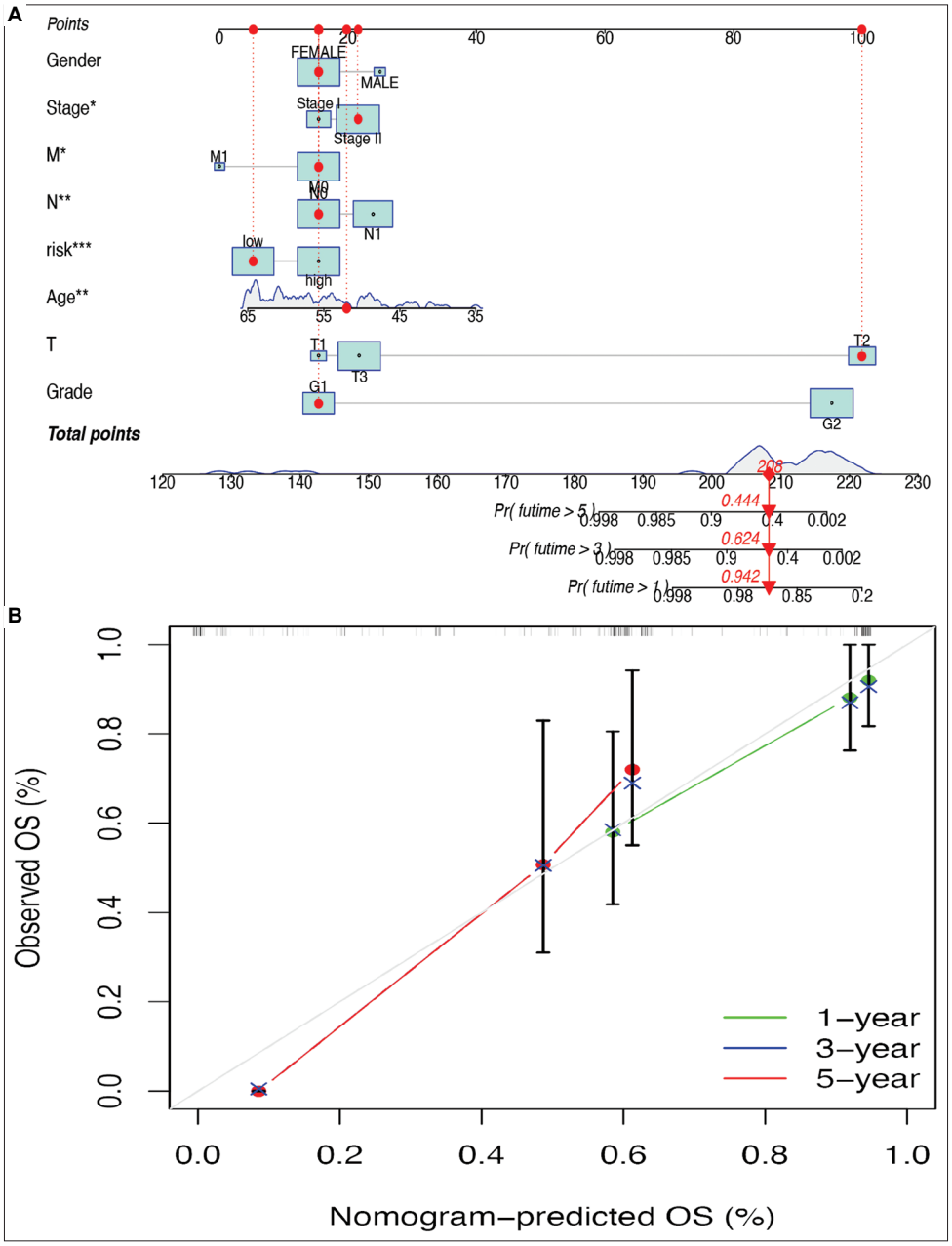

Figure 11. (A) Probabilities of 1-, 3-, and 5-year survival predicted by the nomogram. (B) Calibration plot of the nomogram predicting the probability of 1-, 3-, and 5-year survival.

3.4. Estimation of the tumor immune microenvironment and cancer immunotherapy response by a prognostic model

Immune function analysis was first performed (Figure 7A) and the differences in immune function between high- and low-risk groups were identified. Next, to explore the underlying molecular mechanisms of the m6A-based model, we performed Gene Ontology (GO) enrichment analysis, revealing the involvement of many immune-related biological processes (Figure 7B). An analysis of immune escape and immunotherapy was also performed to find out whether there are differences between high-risk and low-risk groups when receiving immunotherapy (Figure 7C). Mutational data were analyzed and summarized using the maftools package in R studio software. Mutations were stratified according to variant effect predictors. Figure 7D and Figure 7E show the top 20 most frequently altered driver genes between

high-risk and low-risk subgroups. A differential analysis of tumor mutational burden was then performed and the tumor mutational burden (TMB) was then calculated from TGCA somatic mutation data. The low-risk group had lower TMB than the high-risk group (Figure 7F). Next, a survival analysis of TMB was performed. Figure 8A shows that the survival of the low TMB group was better than that of the high TMB group, and then combined with the TMB with the patient risk score for survival analysis, Figure 8B shows that patients with low TMB and low-risk score were found to have a higher probability of survival.

3.5. Identification of potential drugs for prognostic models

To explore potential drugs for the treatment of PAAD patients with our prognostic model, we used the pRRophetic algorithm to estimate treatment response based on the half-maximal inhibitory concentration (IC50) of each drug in the Genomics of Cancer Drug Sensitivity (GDSC) database. We screened for six drugs with significantly different estimated IC50s between the high- and low-risk groups, and the low-risk group was found to be more sensitive to most of the potential drugs. Figure 9 shows six potential drugs that can be used for further analysis of PAAD patients.

3.6. Independent prognostic analysis of prognostic models and assessment of clinical features of PAAD

We performed univariate and multivariate Cox regression analyses to assess whether risk models for m6A-related LncRNAs had independent prognostic features of PAAD. Based on Figure 10A, first in the univariate Cox regression analysis, the HRs for the risk score and 95% confidence interval (CI) were 1.181 and 1.097–1.271, respectively ($p < 0.001$). Based on Figure 10B, HR was 1.162 in multivariate Cox regression analysis, 95% CI was 1.074–1.257 ($P < 0.001$). A concordance index analysis of the risk score was then performed and it was found that the concordance index of the risk score was consistently greater than other clinical factors over time, suggesting that the risk class could better predict the prognosis of PAAD patients (Figure 10C). Thereafter, the area under the ROC curve (AUC) analysis of risk grades was performed (Figure 10D and E), and the AUCs of risk score grades were also shown to be higher than those of other clinical features, indicating that the prognostic risk model constructed in this study was relatively reliable.

3.7. Construction and evaluation of prognostic nomograms

Nomograms including risk classes and clinical characteristics to predict 1-, 3-, and 5-year survival in PAAD patients were constructed (Figure 11A). Based on the correlation plots, the observed and predicted rates of survival in PAAD patients at 1, 3, and 5 years showed good agreement (Figure 11B).

4. Discussion

Pancreatic cancer is the main cause of cancer-related death worldwide and has been a serious threat to human life and health due to its insidious onset, strong invasiveness, poor prognosis, and high mortality rate[2,18,19]. Through further research, it has been found that the disorder of m6A methylation modification regulation may affect the processing, degradation, and translation of mRNA, resulting in the activation of oncogenes and the inactivation of tumor suppressor genes, and the occurrence, development, and drug resistance of malignant tumors. The occurrence of m6A is closely related, and m6A changes play a crucial role in carcinogenesis and tumor progression[8].

M6A plays a post-transcriptional modification role in eukaryotic mRNAs and lncRNAs, such as in regulating mRNA transcription, splicing and translation, as well as affecting the structure and function of lncRNAs with extensive regulatory roles[11]. M6A regulators can modify specific lncRNAs, and lncRNAs can maintain malignancy in various tumors through transcriptional, epigenetic, and post-transcriptional levels[10,20]. The role of m6A-regulated lncRNAs may be critical for the proliferation and migration of cancer cells[11]. Studies have reported that m6A methylation modification of lncRNA can affect the occurrence and development of tumors, and m6A modification can also affect the formation of RNA-DNA triple helix, in which one lncRNA binds to this series through the Hoogsteen base pair in the main groove of double-stranded DNA. In addition, m6A may also affect the reciprocal site between lncRNA and specific DNA[21,22]. Both m6A and lncRNA are important regulators of PAAD occurrence. However, studies on their roles and biological mechanisms in PAAD progression are still relatively lacking[13,17]. In this study, an independent prognostic model based on m6A-related lncRNA was constructed, inspired by the functions of m6A and lncRNA in PAAD.

In this work, 14056 m6A-associated lncRNAs were identified from the TCGA dataset to explore the prognostic functions of m6A-associated lncRNAs. After confirming the prognostic value of m6A-related lncRNAs in the TCGA dataset, five of them were selected to construct m6A-related lncRNA prognostic models to predict the survival of PAAD patients. Model validation for clinical grouping was also performed, the risk scores for each patient in the training group and across the entire set were calculated, and principal component analysis was performed to validate the prognostic model, all of which

demonstrated the accuracy of the prognostic model. PAAD patients were divided into a low-risk group and a high-risk group according to the median prognostic risk level. It was found that the high-risk group had poorer survival than the low-risk group. The model validation results of clinical grouping showed that the model we constructed was more suitable for patients who are 65 years old and below, female patients, and PAAD patients with tumor stage I and II. Multivariate Cox regression analysis showed that the m6A-related lncRNAs prognostic model was an own risk factor for survival. ROC analysis showed that the model outperformed traditional clinical features in predicting survival in PAAD patients. In addition, a nomogram was constructed showing the agreement between the 1-, 3-, and 5-year prognostic model prediction rates for PAAD patients. In terms of the accuracy of the prognostic model based on m6A-related lncRNAs in predicting patient survival, the prediction model can provide a certain basis for subsequent research to identify new biomarkers.

TMB is the total number of somatically encoded mutations associated with the emergence of neoantigens that trigger antitumor immunity[23,24]. Studies have reported that patients with low-risk endometrial cancer have higher TMB and are more sensitive to chemotherapy than patients with high-risk scores[25]. Here, we found that TMB in the low-risk group was lower than that in the high-risk group and then performed a survival analysis of TMB, finding that the low – TMB group had better survival than the high TMB group. Risk scores were used for survival analysis and found that patients with low TMB and low-risk scores had better survival. However, our prognostic model showed that there was no significant difference between high- and low-risk groups when receiving immunotherapy in the analysis of immune escape and immunotherapy, which is probably due to limited samples. More samples can be used in future studies. The tumor microenvironment can regulate the biological properties of tumor cells such as chemotherapy resistance through metabolism and other means. Six drugs with significantly different estimated IC50s were screened out between the high- and low-risk groups. The low-risk group was found to be more sensitive to most potential drugs, the discovery of which may provide new insights into the subsequent treatment of patients with PAAD ideas. Pathological stage is a decisive factor for the diagnosis and prognosis of PAAD[26]. The current staging is not precise in providing reliable predictions and reflecting the heterogeneity of PAAD. Therefore, it is critical to explore new potential predictive markers and immunotherapeutic agents. The m6A-related lncRNA prognostic model established in this paper provides a new idea for predicting the survival of PAAD patients. However, there are some shortcomings and limitations in this study, the biological mechanisms of m6A-related lncRNAs have not been fully elucidated. In the future, the accuracy of this model will be verified with more experiments to explore the role of lncRNA and its interaction with m6A.

5. Conclusion

Understanding the mechanism of m6A-related lncRNA in the development of PAAD may provide new ideas for the prognosis and treatment of pancreatic cancer patients. Our study provides new clues and ways for survival prediction in PAAD patients and may help to elucidate the process and mechanism of regulation between m6A and lncRNA.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest. The authors declare that they have no conflicts of interest.

Author contributions

Conceptualization: Yiyang Chen, Xi Ou
Formal analysis: Yiyang Chen, Yiju Gong
Writing – original draft: Yiyang Chen, Wanbang Zhou
Writing – review & editing: Yiyang Chen, Yiju Gong

All authors contributed to the article and approved the submitted version.

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Not applicable.

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Availability of data

The datasets presented, in this study, can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article.

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Tumor Discovery

REVIEW ARTICLE

Facts and challenges of immunotherapy in triple-negative breast cancer

Xuehai Wang[1], Fengxu Wang[1], Weiyi Xia[1], Siyuan Deng[1], Hongxiang Zhang[2], and Xinyuan Zhao[1]*

[1]Department of Occupational Medicine and Environmental Toxicology, Nantong Key Laboratory of Environmental Toxicology, School of Public Health, Nantong University, Nantong 226019, China
[2]College of Environment and Resources, Southwest University of Science and Technology, Mianyang, Sichuan, 621010, China

Abstract

Triple-negative breast cancer (TNBC) is an aggressive but common cancer subtype in clinical practice. Immune activation has been observed in a subgroup of TNBC, suggesting that immunotherapy may be a potential therapeutic option. With the widespread use of monotherapy, specific immune checkpoint inhibitors (ICIs) such as avelumab, pembrolizumab, and atezolizumab have made significant contributions to improving outcomes in both early and advanced TNBC. In addition, the expressions of immune regulators such as cytotoxic T-lymphocyte-associated protein 4, programmed cell death 1 (PD-1), and programmed cell death-ligand 1 (PD-L1), which are influenced by tumor-infiltrating lymphocytes (TILs), are also critical factors in determining the effect of immunotherapy in TNBC. This review focuses on the updates on the biological underpinnings of TNBC and the associated treatment advances. We present the current landscape of well-known immune regulators and widely used ICIs for TNBC and highlight the future directions that are significant for further improving the efficacy and effect of targeted therapeutic strategies to immunotherapy in TNBC and more reliable prognostic predictions for tailored therapy in the future.

Keywords: Triple-negative breast cancer; Immunotherapy; Immune checkpoint inhibitors; Programmed cell death 1/Programmed cell death-ligand 1; Cytotoxic T-lymphocyte-associated protein 4

***Corresponding author:**
Xinyuan Zhao
(zhaoxinyuan@ntu.edu.cn)

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1. Introduction

Fifteen to twenty percentages of all human breast cancers (BCs) are triple-negative breast cancer (TNBC). TNBC is characterized by the absence of expression of human epidermal growth factor receptor 2 (HER2), estrogen receptor (ER), and progesterone receptor (PR). TNBC frequently exhibits aggressive characteristics, including early recurrence and metastasis[1]. With regard to overall survival (OS), if a patient is found to have stage 1 TNBC, the 5-year survival rate of the patient is nearly 94.7% due to good immune condition and nutrition absorption. The 5-year survival rate of patients with stage 2 TNBC, where the cancer continues to spread but is still confined within the breast or has only affected adjacent lymph nodes, is about 86.37%. In stage 3 TNBC, the cancer has expanded past the tumor's local vicinity and may have even infiltrated adjacent muscles

and lymph nodes, but it has not yet reached distant organs. The 5-year survival rate of patients with stage 3 TNBC is 84%. Stage 4 BC indicates that the cancer has metastasized (spread to other areas of the body), and patients with stage 4 TNBC have only about a 10% chance of survival[2,3]. After all, TNBC is curable when it is diagnosed in the first three stages. The life expectancy and survival rate for stage 3 TNBC are constantly improving[4,5]. At present, there are many treatments available for BC, including surgery, chemotherapy, targeted therapy, and radiotherapy, among which chemotherapy is the primary systemic treatment for the majority of metastatic TNBC (mTNBC) patients[6]. However, responses are frequently transient, and patients have median OS of 12 – 18 months. Moreover, traditional chemotherapy drugs, including paclitaxel, anthracycline, and alkylating agents, are likely to cause side effects and systemic toxicity[4,7]. Therefore, the demand for better therapeutics is increasing.

Based on the expression of ER, PR, and HER2, BC can be classified into four intrinsic subtypes: luminal A, luminal B, HER2+, and TNBC. In most cases, these subtypes have specific immunological characteristics, with different expression levels of tumor-infiltrating lymphocytes (TILs)[8]. In recent years, researchers have found that BC is immunogenetic regardless of its subtype. Lymphocyte-predominant BCs that have stromal or intratumorally lymphocytes make up more than 50 – 60% of the tumor tissue[9,10]. Given that immunotherapy has improved survival in other solid tumors, it may also be a viable option for TNBC treatment. Immune checkpoint inhibitors (ICIs), which inhibit immunosuppressive receptors such as programmed cell death 1 (PD-1) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) to increase the cytotoxicity and proliferation of tumor-infiltrating cells, are the most effective immunotherapy drugs. ICIs, such as pembrolizumab, nivolumab, atezolizumab, durvalumab, and avelumab[11], which are monoclonal antibodies against PD-1, programmed cell death-ligand 1 (PD-L1), and CTLA-4, have produced long-lasting responses in a variety of tumor types[12-15].

Compared with other subtypes, TNBC is more likely to react to immunotherapy due to a number of factors. First off, TNBC contains higher levels of TILs than other BC subtypes, which have been found to be associated with more significant responses to ICIs and a better prognosis for TNBC in its early stages[16]. Second, TNBC has significantly different levels of PD-L1 expression on both immune and cancer tissues[17], making it a direct target for ICIs and correlating with how well those treatments work in treating other malignancies[4]. Third, a better anti-tumor immune response has been mounted by neoantigen-specific T-cells when TNBC has a notable frequency of non-synonymous gene mutations, which lead to tumor-specific neoantigens[16]. These neoantigen-specific T-cell responses can be amplified by ICIs[17,18].

This review offers a framework for comprehending the most recent clinical data relating to immune checkpoint blockade (ICB) and other new immunotherapy drugs for TNBC. Future directions for the development of immunotherapy in TNBC are also explored, along with the development of immunotherapy biomarkers (Tables 1 and 2).

2. Immunotherapy in triple-negative breast cancer

2.1. Triple-negative breast cancer characteristics

TNBC accounts for 15 – 25% of all BCs and is widely recognized as the worst BC among all the subtypes of BC, posing a huge threat to patients diagnosed with BC[1]. TNBC can be classified into four robust subtypes based on their different transcriptomic characteristics: basal-like (BL), immunomodulatory (IM), mesenchymal stem-like (MSL), and luminal androgen receptor (LAR)[30]. A large number of studies have indicated that age, sex, and even race can be risk factors of TNBC. According to research, BRCA and basal TNBC, as well as apocrine and neuroendocrine TNBC, are more common in younger and older women compared to the same age groups in men. It has been documented that Hispanic and African American women are at a higher risk of TNBC and have a poorer prognosis than other populations. In a case study, there was a 2.5% increased risk of TNBC in 187 TNBC patients who had taken oral contraceptives for more than a year, the risk of TNBC was 4.2% for women under the age of 40, and it was discovered that the risk rose as the duration of oral contraceptive use increased[17]. In the United States, TNBC accounts for 12% of BC cases, with 8 – 16% 5-year survival rate.

Other than the four robust subtypes of TNBC, which can be detected at the transcriptomic level, there are four discrete subtypes: LAR, mesenchymal (MES), basal-like immune suppressed (BLIS), and basal-like immune activated (BLIA)[18]. LAR represents TNBC tumors with the lowest genomic complexity, with mutations in phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), AKT serine/threonine kinase 1 (*AKT1*), neurofibromatosis type 1 (*NF1*), GATA binding protein 3 (*GATA3*), and cadherin-1 (*CDH1*)[12-15]. The mesenchymal subtype is characterized by lower genomic complexity and activation of PI3K pathway[31]. BLIA represents the majority of TNBCs with a complex genomic profile, having *TP53* mutations in more than 90% of cases

Table 1. Avelumab, pembrolizumab, and atezolizumab in TNBC treatment

Antibody	Target	Patient population	Sample size	ORR (%)	References
Avelumab	PD-L1	Uncertain breast cancer	168	3.0	Dirix *et al.*[11]
		PD-L1-positive breast cancer	12	16.6	
		PD-L1-negative breast cancer	124	1.6	
		Uncertain TNBC	59	5.2	
		PD-L1-positive TNBC	9	22.2	
		PD-L1-negative TNBC	39	2.6	
Pembrolizumab	PD-1	PD-L1-positive TNBC	27	18.5	Nanda *et al.*[19]
		Uncertain TNBC	170	5.3	Adams *et al.*[20]
		PD-L1-positive TNBC	105	5.7	
		PD-L1-negative TNBC	64	4.6	
		PD-L1-positive TNBC first line	84	21.4	Adams *et al.*[20]
Atezolizumab	PD-L1	Uncertain TNBC	115	10.0	Emends *et al.*[21]
		PD-L1-positive TNBC	91	11.0	
		PD-L1-negative TNBC	21	0.0	
		Uncertain TNBC first line	21	24.0	
		Uncertain TNBC ≥ second line	94	6.0	

ORR: Objective response rate, PD-1: Programmed cell death 1, PD-L1: Programmed death-ligand 1, TNBC: Triple-negative breast cancer

Table 2. Availability of immunotherapy for prevalent cancers

Cancer type	Availability
Bladder cancer	Immune checkpoint inhibitors, T-cell transfer therapy, monoclonal antibodies, treatment vaccines, and immune system modulators[22]
Breast cancer	Immune checkpoint inhibitors, monoclonal antibodies, treatment vaccines, and immune system modulators[23]
Cervical cancer	Immune checkpoint inhibitors, therapeutic vaccines, engineered T-cells, and antibody-drug conjugates[24]
Colorectal cancer	Immune checkpoint inhibitors and monoclonal antibody therapies[25]
Esophageal cancer	Immune checkpoint inhibitors and monoclonal antibody therapies[26]
Head and neck cancer	Immune checkpoint inhibitors[27]
Kidney cancer	Interleukin-2, alpha-interferon, and immune checkpoint inhibitors[28]
Leukemia	Allogeneic bone marrow transplant, therapeutic cancer vaccines, T-cell therapies, monoclonal antibody therapies, and donor lymphocyte infusions[29]
Glioblastoma	No
Ovarian cancer	No

and a high frequency of homologous recombination DNA repair deficiency (HRD). BLIS also shows a high mutation rate in *TP53*, complex genomic profiles, and an HRD-associated signature but are associated with significantly lower TILs[32]. The most noticeable feature about these subtypes is that BLIA and BLIS subtypes are, respectively, associated with the best and the worst disease-free survival. To make a more vivid comparison among the subtypes, the LAR subtype displays mutations similar to those detected in luminal B cancers, and its microenvironment is described as "cold," with low TILs, in comparison with the "desert" microenvironment in the MES subtype and the "hot" microenvironment in the BLIA subtype[33]. However, it should be noted that in these gene expression classification systems, the vast majority of TNBCs analyzed were of high grade; hence, it remains unclear as to how the low-grade forms described above would fit into this taxonomy or if these low-grade forms would constitute completely different entities at the transcriptomic level[34].

The risk factors of TNBC are discussed below. The first is related to age, in which 80% of BC cases (including TNBCs) are older than 50 years old[6]. Due to different sex hormonal stimulation, female sex is considered a higher risk for TNBC compared to male sex. In addition to these two factors, race is also associated with TNBC, in which the incidence of TNBC remains high among Caucasian non-Hispanic women[35]. With regard to breast tissue density, as per clinical practice, breasts can be categorized into low-density breasts, fatty breasts, and high-density breasts[36]. In postmenopausal and premenopausal women, breast density affects the risk of cancer, that is, the higher the

density, the higher the risk of BC[37]. Breast tissue density screening could be a promising and quick approach for rational surveillance. According to several epidemiological studies, obesity is a potential risk factor for BC[38,39]. Hence, engaging in physical activity is considered the best way to prevent BC. Alcohol and alcoholic beverages can also increase the risk of malignancy[40].

2.2. Triple-negative breast cancer microenvironment

The tumor microenvironment (TME) contains various cell types, including fibroblasts, TILs, and lymphatic vascular channels. The active interaction between tumor cells and the microenvironment affects the pathogenesis and development of tumor. Research has indicated that high levels of TILs, especially in the IM subtype, are associated with better prognosis and response to chemotherapy in both neoadjuvant and adjuvant contexts[1,4]. Later, research has revealed that variations in gene overexpression of IM and MSL subtypes are derived from the TME, including infiltrating immune cells and tumor-associated mesenchymal tissue, respectively[41,42]. Intriguingly, and in agreement with the aforementioned findings, these genes are not expressed in cell lines when tests are conducted *in vitro*, where the microenvironment is absent. It is evident that TME has a significant influence on the development of tumor as well as the response and resistance to treatment. Furthermore, the elevated expression of immune regulators such as CTLA4, PD-1, and PD-L1 in TNBC, brought on by lymphocyte infiltration of the tumor, is likely linked to a response to ICIs. Several studies have purported the possibility that TILs may be a marker for improved survival outcomes[43-45]. All the preceding evidence suggests that focusing on TME in TNBC and further exploring the biomarker landscape are promising efforts for better immunotherapy.

2.3. Specific biomarkers

Due to the underlying heterogeneity of TNBC, there is a need for efficient biomarkers that can guide doctors in determining the best course of action. Therapeutic trials have been conducted on several suggested biomarkers for TNBC, with limited clinical benefits so far. Breast cancer gene (*BRCA1*/*BRCA2*) mutations have been found to be predictive of the effectiveness of poly (ADP-ribose) polymerase (PARP) inhibitors, and changes to other homologous recombination-related genes appear promising in this context[46-49]. It is possible to use the expression of PD-L1 protein in either immune cells (ICs) or tumor cells, or both, as a biomarker to predict how well an immune checkpoint inhibitor would work[15]. TILs are also considered an important prognostic factor in TNBC. Up to 15 studies have shown that 11% (median; range, 5 – 26%) of breast cancers are lymphocyte-predominant breast cancers (LPBCs), among which TNBC accounts for the highest incidence (20%; range, 4 – 37%)[50]. Moreover, $CD8^+$ T-cell infiltrates have been observed in 60% of TNBC cases[41]. Various tests that employ different antibodies and scoring methods are commercially available. There is an ongoing debate over the optimal assay for TNBC and whether the findings hold for all ICIs.

2.4. Drugs

ICIs, especially for CTLA-4, PD-L1, and PD-1, have made great contributions to cancer therapy. The five drugs in TNBC immunotherapy include avelumab, pembrolizumab, and atezolizumab, ipilimumab, and tremelimumab.

2.4.1. Programmed cell death 1/programmed cell death-ligand 1 inhibitors

Avelumab, a complete monoclonal antibody of the isotype IgG1 that binds to PD-L1 and prevents binding to its receptor PD-1, acts as a checkpoint inhibitor, and is being utilized in the immunotherapy for various types of advanced or metastatic cancers[51,52]. In the TNBC subgroup treated with avelumab, there were three partial responses (PRs), giving TNBC patients an objective response rate (ORR) of 5.2% (95% CI, 1.1 – 14.4%). The disease control rates (DCRs) were 28% (47/168) and 31% (18/58) for the overall population and the TNBC subgroup, respectively. Both the overall population and the TNBC subgroup showed a tendency toward higher ORRs in patients with PD-L1 expression in tumor-associated ICs (10% cutoff), with ORRs of 16.7% (2/12 patients) and 22.2% (2/9), respectively, for PD-L1-positive disease and 1.6% (2/124 patients) and 2.6% (1/39 patients), respectively, for PD-L1-negative disease[53].

A humanized antibody, pembrolizumab, is used in cancer immunotherapy to treat melanoma, lung, head and neck, stomach, cervical, and breast cancers, as well as Hodgkin lymphoma. Pembrolizumab is slowly injected into a vein. The IgG4 isotype antibody blocks the defense mechanism of cancer cells, thus enabling the immune system to eliminate them. Pembrolizumab targets the lymphocyte PD-1 receptor and functions by concentrating on the PD-1/PD-L1 biological pathway, which is present in some cancer cells and immune cells in the body[6,54-61]. The PD-1 antagonist pembrolizumab, in the KEYNOTE-012 trial, which studied the safety and antitumor efficacy of pembrolizumab monotherapy in patients with advanced PD-L1-positive solid tumors, was initially assessed in PD-LI-positive advanced TNBC patients. PD-L1 expression was prescreened in 111 patients with advanced TNBC, in which 58.6% of them tested positive for PD-L1. The median number of prior therapies for advanced illness

was two among 32 treated TNBC patients (range, 0 – 9). Of these, 27 patients had their clinical responses evaluated. With 1 complete response (CR) and 4 PRs, the ORR was 18.5% (95% CI, 6.3 – 38.1%), and the DCR was 25.9% (95% CI, 11.1 – 46.3%)[62].

By preventing the interaction of PD-L1 with PD-1 and CD80 receptors (B7-1Rs), atezolizumab can be used to treat dysplastic carcinoma, hepatocellular carcinoma (HCC), non-small-cell lung cancer (NSCLC), small-cell lung cancer (SCLC), and TNBC[63,64]. Atezolizumab is a monoclonal antibody of the IgG1 isotype that has been fully humanized and engineered to target the protein PD-L1[13,65-67]. In the PCD4989g trial, atezolizumab was tested in patients with advanced malignancies[68], including 116 patients with advanced TNBC, 115 of whom had an objective response assessed. After the enrollment of the initial 25 patients, the eligibility was changed to permit the enrollment of patients with any PD-Ll status. These patients displayed PD-LI in IC, occupying <5% of the tumor area. With 58% of patients having received at least two prior lines of therapy for an incurable illness, the enrolled patients were severely treated[68,69].

2.4.2. Cytotoxic T-lymphocyte-associated protein 4 inhibitors

Ipilimumab is a monoclonal antibody that works to activate the immune system by targeting CTLA-4, a protein receptor that downregulates the immune system. It boosts the immune response against cancer cells and prevents the inhibitory interruption of cytotoxic T-lymphocytes (CTLs), which can recognize and destroy cancer cells[70]. In a study on early-stage BC, 12 of 18 women received a single dose of neoadjuvant ipilimumab alone or with additional cryoablation; the other six patients received cryoablation alone. T-cell density was found to be significantly correlated with TIL count by hematoxylin and eosin (H&E). It was shown that about 5/6 patients who received ipilimumab alone had increased T-cell density in contrast to the decrease in the cryoablation group[71,72].

Tremelimumab blocks the binding of antigen-presenting cell ligands B7.1 and B7.2 to CTLA-4, resulting in the inhibition of B7-CTLA-4-mediated downregulation of T-cell activation. Tremelimumab has been evaluated in various types of tumors. Experiments with tremelimumab in combination with exemestane have been carried out. Among 26 patients who received tremelimumab (3 – 10 mg/kg) and exemestane (25 mg/kg daily), five patients developed dose-limiting toxicity when the dose of tremelimumab with 25 mg/day exemestane was about 6 mg/kg Q90D, four of which were diarrhea. Up to 42% of patients had stable disease for at least 12 weeks. The percentage of $CD4^+$ and $CD8^+$ T cells expressing inducible T-cell costimulator (ICOS) increased in all patients[73].

2.4.3. Neoadjuvant ipilimumab

These groundbreaking studies offer solid proof in favor of using PD-1/PD-L1 and CTLA-4 inhibitors in both early and advanced TNBC. The FDA has authorized the use of pembrolizumab in conjunction with chemotherapy for the treatment of PD-L1-positive advanced TNBC, and health authorities now recommend the combination of atezolizumab and nab-paclitaxel[55]. To further understand the immunobiology of both early and late TNBC, well-controlled translational studies may be conducted using the data sets and tissue samples from these trials[49,60]. By identifying TNBC as a tumor that can react to immunotherapy, these studies collectively pave the way for the testing of cutting-edge ideas that can successfully harness the immune system to improve clinical outcomes for patients with this arduous condition (Table 3).

3. Challenges of immunotherapy in triple-negative breast cancer

3.1. Unclear mechanism of tumor-infiltrating lymphocytes

It is essential to consider whether the number of TILs expressed in the primary tumor and the metastatic sites can affect the prognosis of patients with TNBC. Another issue is whether the heterogeneity of TILs at the original sites, the occurrence of residual invasive disease (RD) following the completion of neoadjuvant chemotherapy (NAC), and the metastatic locations can influence the choice of follow-up therapy options in TNBC[2,54]. Despite the fact that the discovery of biomarkers has given individuals who are looking to advance their skills an effective tool, one of the main limitations of the use of TILs at the moment is their reliance on manual measurement, which is subject to potential human error[41]. Surprisingly, there are many opportunities to employ computational techniques that extract spatial-morphologic predictive elements, thus making it possible for computer-aided diagnostics.

We, now, need to figure out how to assess TILs in combination with other biomarkers to direct a more focused course of treatment, as they have been established as distinct biomarkers in the early TNBC. In addition, it is still worthwhile to advocate the current belief that TILs serve as the starting point for the expression of additional biomarkers[16,51].

In terms of immunomodulatory mechanisms, whether negative immunomodulatory regulation, as part of a standard feedback loop, has a positive and persistent

Table 3. Randomized phase III clinical trials of PD-1/PD-L1 blockade for metastatic TNBC

Trial	Sample size	Key eligibility	Intervention	ORR	Median PFS	Median OS	Reference
IMpassion130 Randomized 1:1, double-blind, placebo-controlled	902	1st line mTNBC TFI ≥ 12 months Any PD-L1 status	Atezolizumab + nab-paclitaxel versus placebo + nab-paclitaxel	ITT 56% versus 46% PD-L1-positive IC 59% versus 43%	ITT 7.2 versus 5.5 months PD-L1-positive IC 7.5 versus 5.0 months	ITT 21.0 versus 18.7 months PD-L1-positive IC 25.4 versus 17.9 months	Schmid *et al.*[69] Miles *et al.*[74]
IMpassion130 Randomized 2:1, double-blind, placebo-controlled	651	1st line mTNBC TFI C mTNBC :1, Any PD-L1 status	Atezolizumab + paclitaxel versus placebo + nab-paclitaxel	PD-L1-positive IC 63.4% versus 55.4% ITT 53.6% versus 47.5%	PD-L1-positive IC 6.0 versus 5.7 months ITT 5.7 versus 5.6 months	PD-L1-positive 28.3 versus 22.1 months I ITT 22.8 versus 19.2 months	Miles *et al.*[74]
KEYNOTE-119 Phase III, randomized 1:1, open-label	622	2nd or 3rd line mTNBC Prior A and T Any PD-L1 status	Pembrolizumab monotherapy versus chemotherapy of physician's choice*	ITT 9.6% versus 10.6% CPS ≥ 1 12.3% versus 9.4% CPS ≥ 10 17.7% versus 9.2% CPS ≥ 20 26.3% versus 11.5%	ITT 2.1 versus 3.3 months CPS ≥ 1 2.1 versus 3.1 months CPS ≥ 10 2.1 versus 3.4 months CPS ≥ 20 3.4 versus 2.4 months	ITT 9.9 versus 10.8 months CPS ≥ 1 10.7 versus 10.2 months CPS 2 months versus 11.6 months CPS ≥ 20 months versus 12.5 months	Oki *et al.*[75]
KEYNOTE-355 Phase III, randomized 2:1, double-blind, placebo-controlled	847	1st line mTNBC TFI C mTNBCrandoany PD-L1	Pembrolizumab + chemotherapy# versus placebo + chemotherapy	NR	ITT 7.5 versus 5.6 months	NR	Cortes *et al.*[76]

A: Anthracycline, CPS: Combined positive score, IC: Immune cell, ITT: Intent-to-treat, mTNBC: metastatic TNBC, NR: Not reported, ORR: Objective response rate, OS: Overall survival, PD-L1: Programmed death-ligand 1, PFS: Progression-free survival, T: Taxane, TFI: Treatment-free interval. *Chemotherapy of physician's choice could be capecitabine, eribulin, or gemcitabine. #Chemotherapy of physician's choice could be paclitaxel, nab-paclitaxel, or gemcitabine + carboplatinum

effect on tumor immune response is worth discussing. Furthermore, more exploratory work needs to be done to determine whether the possible mechanism above potentially defines a more immunogenic tumor. At the same time, we should continue to focus on the heterogeneity of TILs, the subpopulation classification of T cells, and how their respective molecular pathways regulate immunity[44,73].

3.2. Unpredictable personal benefits

For the purpose of selecting patients who are most likely to benefit from immunotherapy and the development of combination treatments to overcome drug resistance, tumor molecular profiling is significant. Through gene expression profiling analysis of TNBC tumor samples, abnormal cell cycle-regulating and DNA repair-related gene expression has been observed in the BL1 subtype[77]. Possible therapeutic drugs for the BL1 subtype include PARP inhibitors and genotoxic agents; BL1 patients are usually sensitive to cisplatin treatment. On the other hand, the BL2 subtype has abnormal activation of signaling pathways, such as the epidermal growth factor receptor (EGFR), mesenchymal epithelial transition factor (MET), nerve growth factor (NGF), Wnt/β-catenin, and insulin-like growth factor-1 receptor (IGF-1R) pathways, and the potential targeted therapeutic drugs include mammalian target of rapamycin (mTOR) inhibitors and growth factor inhibitors (lapatinib, gefitinib, and cetuximab)[64]. Meanwhile, the IM subtype has significantly enriched immune cell-associated genes and signal transduction pathways, such as the Th1/Th2, NK cell, B-cell receptor, dendritic cell (DC), T-cell receptor, interleukin (IL)-12, and IL-7 pathways[78]; thus, the IM subtype is highly similar to medullary carcinoma of the breast. PD1, PDL1, CTLA-4, and other immune checkpoint inhibitors are recommended for the treatment of patients with breast cancer of the IM subtype. The M subtype, on the other hand, has highly activated cell migration-related signaling

pathways (regulated by actin), extracellular matrix-receptor interaction pathways, and differentiation pathways (Wnt pathway, anaplastic lymphoma kinase pathway, and transforming growth factor [TGF]-β signaling). The M subtype has sarcoma-like or squamous epithelial cell-like tissue characteristics and is prone to developing resistance to chemotherapy drugs[79]. Patients with the M subtype may be treated with mTOR inhibitors or drugs that target epithelial-mesenchymal transition[15]. Compared with the M subtype, the MSL subtype shows a lower expression of cell proliferation-related genes but a higher expression of stem cell-related genes, *HOX* genes, and mesenchymal stem cell-specific markers. Presumably, patients with the MSL subtype can be treated with PI3K inhibitors, Src antagonists, and angiogenesis inhibitors[80]. Compared with other TNBC subtypes, the LAR subtype has a significantly different gene expression profile[81]. This subtype does not express ERs, but has highly activated hormone-related signaling pathways (e.g., steroid synthesis, porphyrin metabolism, and androgen/estrogen metabolism). Notably, androgen receptors (ARs) are highly expressed in breast cancers of the LAR subtype, with messenger ribonucleic acid (mRNA) levels nine times higher than in other TNBC subtypes. Immunohistochemistry has also shown that several metabolic markers of AR and its associated activators (24-dehydrocholesterol reductase [DHCR[24]], activated leukocyte cell adhesion molecule [ALCAM], fatty acid synthase [FASN], FK506-binding protein 5 [FKBP[5]], apolipoprotein D [APOD], prolactin-induced protein [PIP], sterile alpha motif pointed domain-containing ETS transcription factor [SPDEF], and claudin-8 [CLDN[8]]) are highly expressed in the LAR subtype. Therefore, anti-AR therapy is recommended for breast cancer patients with the LAR subtype.

Besides, biomarkers that predict the clinical benefit of immunotherapy in TNBC are also required. PD-L1 expression on immune cells and mismatch-repair deficiency are the only two validated biomarkers that are currently available[82,83]. The majority of patients with mTNBC are PD-L1-negative by the presently authorized SP142 test[83], despite the fact that mismatch repair failure is uncommon in breast cancer but more frequent in the early-stage illness[73]. The variability of PD-L1 expression over time and at metastatic sites[84], the discrepancy between PD-L1 assays, particularly when staining immune cells, the observation that some PD-L1-negative patients respond to ICIs[85], and the recent trials in early disease setting that show little to no correlation of PD-L1 expression with benefit specific to ICIs, such as the KEYNOTE522 and NeoTRIPaPDL1 trials, are additional factors that limit the utility of PD-L1[85,86].

Moreover, the IMpassion130 trial has shown a significant, modest improvement in progression-free survival (PFS) but a marked difference in OS[87]. The subgroup of patients with PDL1 > 1% (185/451 patients) benefited from atezolizumab; a trend toward a higher ORR was observed in patients with PD-L1-positive versus PD-L1-negative ICs in the overall population[69]. With the various outcomes from clinical trials, the focus of further research is on identifying more prognostic biomarkers to demonstrate the benefit for each patient receiving immunotherapy (Table 3).

4. Conclusion

TNBC, compared with other BC subtypes, has a poor prognosis and is still a complicated cancer for immunotherapy to be developed thus far. However, to improve the prognosis, immunotherapy techniques must be used. Research efforts should focus on using the ICB we have in relation to the molecular biology of TNBC to discover mono antibody therapies and other more effective drug combinations. For this highly diverse subtype of BC, personalized medicine appears to be of great importance. When deciding on a treatment plan, tumor molecular profiling should be carried out at the time of diagnosis, after each tumor recurrence or progression, and as needed. The functions of a cluster of biomarkers may be crucial to predicting an individual's response to future TNBC immunotherapy.

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Conflict of interest

The authors declare that they have no competing interests.

Author contributions

Conceptualization: Xuehai Wang, Fengxu Wang, Weiyi Xia
Funding acquisition: Xinyuan Zhao
Investigation: Hongxiang Zhang
Supervision: Siyuan Deng, Xinyuan Zhao
Writing – original draft: Xuehai Wang
Writing – review & editing: Xuehai Wang, Fengxu Wang, Weiyi Xia, Siyuan Deng, Xinyuan Zhao

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ACCSCIENCE PUBLISHING

Tumor Discovery

SECONDARY PUBLICATION

Benefits and harms of screening: Overdiagnosis and anticipatory medicine – A secondary publication

Antonio Zarazaga Monzon[1]*, Ángeles Franco-López[2,3], and Jesús M. Culebras[4]

[1]Department of Surgery, Autonomous University of Madrid, 28049 Madrid, Spain

[2]Radiology Services, Vinalopó and Torrevieja Hospitals, Spain

[3]University by ANECA, Alicante, Spain

[4]Royal Academy of Medicine of Valladolid and IBIOMED, University of León, León, Spain

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***Corresponding author:**
Antonio Zarazaga Monzon (ant.zarazaga@gmail.com)

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Abstract

The treatment of breast cancer has changed markedly since the publication of works that recommend screening for the early diagnosis of breast cancer. Retrospective reevaluations have revealed errors in screening; moreover, advances in oncological therapy and a better understanding of the disease have raised doubts toward the efficacy of these procedures, which might also cause side effects alongside the risk of overdiagnosis and overtreatment. On the other hand, the lack of information or even misinformation might cause confusion among the potential beneficiaries of these procedures, particularly the patients. These procedures are constantly being recommended by institutions, but the possible risks accompanied by these procedures are often not explained. It is easy to promote mammography screening if the majority believe that it reduces the risk of breast cancer and saves lives. Unfortunately, this is not the case. Many critics of screening are now demanding clear and precise explanations of the procedure and emphasizing on the importance of physical examination. Women must make informed decisions before screening by discussing their own risk profile, the possible benefits, and the eventual risks and harms of mammogram with their physicians. Women should be classified into two groups: those who would gain potential benefits from the procedure and those whose risks outweigh the benefits. A screening program that clearly does not offer more benefits than risks cannot be implemented by public heath institutions. Providing complete and unbiased information, promoting appropriate care, as well as preventing overdiagnosis and overtreatment would be the best option.

Keywords: Screening; Breast cancer; Mammogram; Overdiagnosis; Anticipatory medicine

1. Introduction

Many researchers have criticized the efficiency of screening. They concur that trials advocating universal screening suffer from biased information on optimal results, use misleading advertising, and minimize or even conceal the negative physical and psychological effects caused by the application of screening in healthy people as well as the lack of information provided to the people.

First, we must differentiate a diagnostic and/or detection procedure from a screening procedure, as well as preventive medicine from anticipatory medicine (primary prevention).

In screening, the individuals included in the process are asymptomatic and have no medical history nor have that they undergone any examination before screening; otherwise, it would be considered a diagnostic procedure.

Regardless of the sensitivity and specificity of each screening procedure, not all of them present the same degree of inconvenience (damage and harm resulting from doing something). In some cases of screening, such as those for breast, colon, and prostate cancer, they are based on imaging test and/or endoscopy, in which risks may arise due to diagnostic errors and subsequent actions. There are other screenings, such as the screening for atheromatous cardiovascular disease, in which the procedure is totally predictive since it is not based on images, but rather the scores obtained through risk adjustment systems that make long-term predictions (up to 10 years), which might eventually lead to potentially harmful and unnecessary pharmacological treatments.

In 1975, Sackett published a paper in *The Lancet*[1] on the discussions and debates between the different roles of screening, case finding, diagnosis, and epidemiological surveys in disease detection. According to Sackett, discussions would improve when participants define the different purposes and characteristics of each procedure, recognize the ideological and intentional differences between the defenders and the critics, and value the quantitative and qualitative differences for decision-making in front of the individual patient or before the community.

While the advocates of screening, generally for irreproachable reasons, have claimed that with the existing evidence and given the current rate of disability and premature death, mass screening programs should be imposed for the detection of citizens with risk factors; methodologists have insisted that screening, like any other unproven health practice, could do more harm than good, and should meet scientific and ethical criteria before being implemented.

Sackett revealed the differences between the advice directed at an individual patient and that directed at a community. A higher level of evidence of efficacy is required to recommend treatment at the community level, especially when patients are solicited through screening. A community cannot be treated as a patient and *vice versa*.

Years later, in 2002, Sackett's displeasure toward the application of this type of medicine became more evident[2]. Sackett claimed that preventive medicine (referring to primary prevention, or as its critics call it, anticipatory medicine) displays all three elements of arrogance. First, preventive medicine is "aggressive" in that asymptomatic individuals are often solicited and instructed on what they have to do to stay healthy; second, preventive medicine is "presumptuous" in that it assumes that its prescriptions always did more good than harm; third, preventive medicine is "despotic" in that it lashes out at anyone who dissents from its recommendations.

Considering the complications arising from overdiagnosis, and especially with overtreatment, Sackett argues that the pledge we must make when we solicit and exhort individuals to accept preventive interventions should be that they will be better off by adopting these measures. Consequently, the assumption that justifies the aggressive assertiveness with which we go after *naïve* healthy individuals must be based on the highest level of evidence. We must be certain that our preventive maneuvering does, in fact, do more good than harm.

A number of studies have demonstrated that the main tool of overdiagnosis, universal screening, is expensive, ineffective, and even dangerous. Therefore, every individual should be informed of the risks, inconveniences, and dangers of each proposed test other than its possible benefits.

However, it seems that apart from these uncertain benefits, political and/or economic cost-effectiveness are some of the advantages of screening, which are enhanced when both objectives coincide.

These premises serve as the foundation for our analysis of a screening that is widely accepted.

2. Breast cancer screening

In a review of five Swedish trials, published in *The Lancet* in 1993[3], it was found that screening reduced breast cancer mortality by 29% (however, as we shall discover later, this was not the case). Despite this, the review has also addressed the need to consider other factors, both beneficial and harmful ones, in addition to mortality, before recommending universal screening. Needless to say, that 29% of successes were, in principle, highly appealing, thus concealing other recommendations.

In reality, this reduction in mortality is equivalent to saving one woman in every 1000 screened over 10 years. The benefit of detection is therefore very small. Translating it into standard language, according to the study, in that 10-year period, four women out of 1000 died from breast cancer, while only three died among those screened. Therefore, the absolute reduction in mortality for breast cancer was only 0.1% (1 in 1000) after 10 years. This 0.1%, using relative risk reduction (RRR), became the 29% cited.

Moreover, those figures were "inflated." Later, reviews have found that the reduction in mortality was in fact

smaller. The most exhaustive evaluation was that of a Cochrane review in 2009[4], which included six studies and 600,000 women. After accounting for the biases identified in those studies, the RRR of mortality was in fact half of the aforementioned (15%), or what amounts to the same thing, that it was necessary to screen 2000 women in ten years (twice as much) for one to benefit compared to the group that was not screened (absolute risk reduction: 0.05%). On the other hand, this benefit was non-existent when evaluating the overall mortality since it was the same in both the groups, which could be ascribed to the consequences resulting from overtreatment in the screening group.

The relevance of overdiagnosis and overtreatment was also acknowledged in consideration of the cumulative risk of false positive results. Overdiagnosis reached 30% that is to say that 10 healthy women (who if there had been no screening would not have been overdiagnosed) were treated unnecessarily, and although no one can say with certainty which women have overtreated tumors, there is certainty about what happens to them: they would have to undergo surgery, radiotherapy, hormonal therapy for 5 years or more, chemotherapy, or a combination of all of these to treat abnormalities that otherwise would not have caused disease[5]. It has been warned that repeated screening increases the risk of overdiagnosis as shown by the risk ranging from about 20–60% after 10 years of mammography screening.

The review revealed for the first time that psychological harm from breast cancer screening is substantial and long-lasting, affecting a large number of healthy women (over 200 women experienced significant psychological harm).

In 2011, the National Breast Cancer Coalition (NBCC)[6], after two exhaustive reviews on screening, concluded that the general impact on mortality is small and that the existing biases in the trials could either "erase it" or "create it."

Mammography, which has many limitations, does not prevent or cure breast cancer. Women should discuss with their physicians their own risk profile, the potential benefits and harms, the complexities of screening mammography, and then make informed decisions about the screening. Women who have symptoms of breast cancer, such as a lump, pain, or nipple discharge, should have a diagnostic mammogram performed.

The update on the Cochrane database review, carried out in 2013[7], found no positive effect of screening on mortality from breast cancer, nor on overall mortality. They believe that due to advances in breast cancer treatment and increased general awareness, the absolute effect of screening was likely to be less than that shown in the trials. In fact, recent studies have suggested that screening is no longer effective[8,9]. This finding has led to the abolishment of screening mammography by the Swiss Medical Council in 2014[10].

The importance of women making informed decision to accept screening or not has been emphasized, and an evidence-based informative booklet that is available in several languages has also been published[11].

In a comprehensive review of scientific literature, published in *The BMJ*, Prasad *et al.*[12] have found that disease-specific mortality is an unreliable proxy for overall mortality. Even when a screening technique lowers disease-specific mortality rates, which is generally rare or only to a slight degree, there are no significant differences in overall mortality. Negative effects of screening may override any disease-specific benefits.

If screening does not reduce the risk of mortality from cancer (including breast cancer), why are screening campaigns so successful?

3. Misinformation and misrepresentations: Misconceptions by women

In 2014, Biller-Andorno and Jüni[10] revealed the enormous discrepancy between women's perceptions of the benefits of mammogram and those expected in reality. Of 1003 women questioned, 71.5% believed that mammogram can reduce the risk of mortality from breast cancer by at least half, while 72.1% believed that it can prevent at least 80 deaths/1000 women screened. Nothing could be further from reality than this.

He concludes that promoting mammography screening is easy if most women believe that it prevents or reduces the risk of breast cancer and saves lives through early detection of aggressive tumors. We would be in favor of mammography screening only if these beliefs were valid. Unfortunately, they are not, and we believe women need to be told that.

4. Incorrect information

Screening advocates and their organizations often emphasize the benefits while omitting information on major harms when providing information materials[7].

In 2016, Gigerenzer[13], in his editorial in *The BMJ*, which is attached to the review by Prasad *et al.*[12], stressed on the influence of language and the persuasiveness of words. Instead of saying "early diagnosis," supporters of screening use the term "prevention." This erroneously suggests that screening lowers the chance of developing cancer. Does this then imply that not getting screened for cancer increases the risk of developing cancer?

Three other instances of how language is used to underline the benefits of screening are as follows: (i) presenting the benefits in relative rather than absolute terms; (ii) comparing increases in 5-year survival rates with decreases in mortality rates; and (iii) showing that the women who are screened by mammography are referred to as patients, who could be healthy people.

5. Marketing and its benefits: Political profitability

The information women receive when they are invited to participate in mammography screening tends to be biased, insufficient, and misleading.

Information on the internet, for instance, on cancer fundraising websites, often omits the harms or portrays them as the benefits.

These invitations generally focus on the benefits of screening, rather than providing information on the proportion of healthy women who are overdiagnosed or overtreated.

When women are invited for mammography screening, the common practice is that when they receive the letter, they are also given an appointment for the examination. This puts pressure on women, and thus, their participation in screening is less voluntary. In some countries, women are even telephoned at home and encouraged to participate, which is also potentially coercive.

Screening is said to reduce a woman's risk of losing her breast. This is a false fact. Instead, screening increases the risk of lumpectomy or mastectomy as a result of overdiagnosis and overtreatment.

6. The collectives

Support groups, organizations, advertising campaigns, community screening events, *etc*., consider universal screening as an advance or a social achievement, without having awareness of the risks of overdiagnosis. Added to this is the fact that the information they receive is incomplete and sometimes false, exaggerating the benefits and concealing the disadvantages and, above all, the risks. They do not understand that in this case, "less is more and more is less." Direct access to "non-suspicious" and independent information, such as that provided by the NBCC[6] or the Nordic Cochrane Center[12], could reassure some sensitivities.

7. Sociopolitical profitability

Although we consider that professionals should be familiar with all publications on the subject, and despite the number of existing screening programs in communities, private medical societies, and organizations, which do not doubt the excellence of the system, we must, in this case, think as follows: "It does not smell rotten in Denmark," but rather it smells like "sardines being pulled up by their own bootstraps". and how can they throw stones, not even sardines, at their own roof?

It is noteworthy that the primary objective of a breast cancer prevention plan in a specific autonomous community is the participation of at least 70% of women who have been invited to participate. If that is the objective, to ensure maintenance budgets, how is screening supposed to be recommended to women in an unbiased manner? On the other hand, there were no assessments for tumor detection, false-positives, adverse events, unnecessary interventions, *etc*. Even the indicator "cancer detection rate within the program" was specified as "Not available" in the findings. Clearly, it takes 10 years and 2000 women to get three!

The last objective, which is the ninth on the list, is to improve the training and knowledge of professionals and the general public on preventive aspects of cancer. However, it does not seem that this objective can be achieved either.

Prasad *et al*.[12] have recommended that health-care providers should be frank about the limitations of screening. The first step public health experts should take is to convey the message that mass screening of healthy people for cancer is not equivalent to health preservation. To say explicitly or implicitly that screening saves lives when there is no evidence to support this claim and much to the contrary undermines confidence toward the medical profession.

8. Conclusion

From an ethical perspective, it would be difficult to justify the implementation of a public health program that clearly does not bring more benefit than harm. Providing clear and unbiased information, promoting appropriate care, and preventing overdiagnosis and overtreatment would be the best option.

Women, physicians, and health-care policymakers should carefully consider the trade-offs when deciding whether to participate in screening programs.

Given all of that, we are not implying that all cancer screening is futile. People with a higher baseline risk of cancer, such as those with a family history of cancer or environmental exposure, may benefit from screening. Similar to Prasad and the NBCC[6,12], we believe that it is advisable to invest money in research for such patients.

It is understandable that some people, even with objective data at hand, still prefer to be screened. There is also much to be debated on concerning who should

be financially responsible for the application of medical procedures that are not based on scientific evidence.

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Editorial disclosure

The paper, after translation, has been edited to adapt it to the format and style of *Tumor Discovery*. The most obvious change to the paper is the removal of Summary section ("Resumen" in original).

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ACCSCIENCE PUBLISHING

Tumor Discovery

CASE REPORT

A case report of aggressive sebaceous carcinoma of the scalp

Sunil V. Jagtap[1]*, Swati S. Jagtap[2], Shefali Mishra[1], Kaushiki Varshney[1], and Shuchita Gaur[1]

[1]Department of Pathology, Krishna Institute of Medical Sciences Deemed University, Karad, Maharashtra, India

[2]Department of Physiology, Krishna Institute of Medical Sciences Deemed University, Karad, Maharashtra, India

Abstract

Malignant pilosebaceous neoplasm of the scalp is a very rare tumor. A 60-year-old man presented with a rapidly enlarging, ulcerated, and firm nodular mass over the scalp for a duration of 3 months. A few months back, the patient noticed a subcutaneous nodule at the same site, and it was reported as sebaceous adenoma on histopathology. The swelling recurred at the same site and was surgically excised and sent for histopathology. A histological diagnosis of sebaceous carcinoma of the scalp was made. On follow-up, there was no recurrence or distant metastasis. Due to the rarity and aggressive behavior of the malignant pilosebaceous neoplasm of the scalp, we present this case along with clinical and histopathological findings.

Keywords: Sebaceous carcinoma; Scalp nodule; Aggressive cutaneous tumor

***Corresponding author:**
Sunil V. Jagtap
(drsvjagtap@gmail.com)

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1. Background

Malignant pilosebaceous neoplasm or sebaceous carcinoma (SC) is a rare tumor of the sebaceous gland. It can be classified into ocular and extraocular types. It commonly affects the head-and-neck region, with the periocular area being the most common site[1]. SC typically presents as a firm subcutaneous nodule that enlarges gradually. The aggressive behavior of this tumor is debatable[2].

2. Case presentation

A 60-year-old man presented with a rapidly enlarging, ulcerated, and firm nodular mass over the scalp for a duration of 3 months. He had no family history of malignancy or other contributory family history. Several months back, he noticed a subcutaneous nodule at the same site and it was diagnosed as sebaceous adenoma on histopathology by a pathology laboratory. After an interval of 2 months, it recurred, following which he underwent wide local excision again.

On examination, a single, nodular swelling over the scalp at the occipital region was observed with surface ulceration, measuring 2.5 × 1.8 cm. It was soft to firm in consistency and adhered to the overlying skin. He had no lymphadenopathy and his systemic examination was normal.

The tumor was surgically excised with wide margins. On gross examination, it was a single nodular mass measuring 2.5 × 1.5 × 1.0 cm with surface ulceration. On cut section, it was firm to hard, greyish-brown, and fleshy (Figure 1).

Histopathological examination (Figures 2 and 3) with hematoxylin and eosin showed neoplastic cells of basaloid, basosquamous, and epidermoid type with varying degrees of differentiation and arranged in irregular lobules and sheets. The intervening stroma was fibrovascular. The tumor lobules had mild pleomorphic cells with hyperchromatic nuclei and a moderate amount of vacuolated or foamy cytoplasm. There was also increased mitoses. In poorly differentiated areas, the tumor cells were highly pleomorphic, hyperchromatic, or vesicular with prominent nucleoli. The neoplastic cells had large multivacuolated foamy cytoplasm. Multifocal epidermal ulceration was also observed. A histological diagnosis of sebaceous carcinoma of the scalp was made.

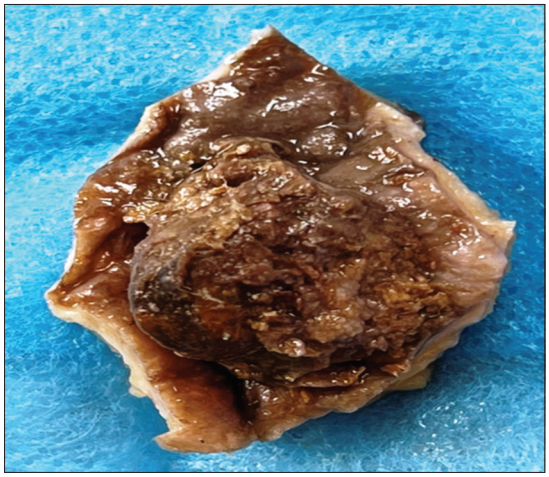

Figure 1. Gross examination showing a nodular mass lesion measuring 2.5 × 1.5 ×1.0 cm with surface ulceration.

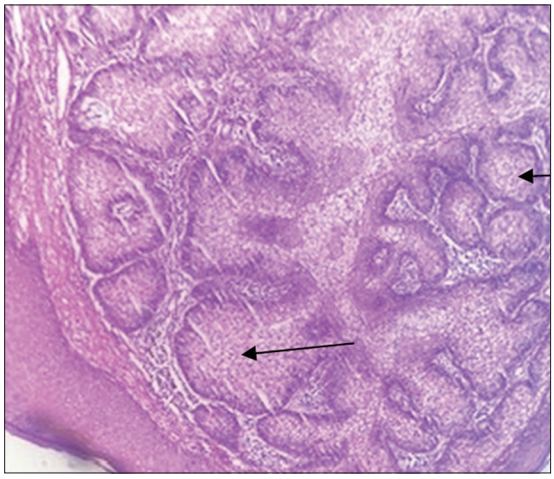

Figure 2. Photomicrograph showing neoplastic cells with various degrees of differentiation and arranged in irregular lobules of atypical sebaceous cells (arrows) (H&E stain, ×40).

On follow-up, the patient was asymptomatic, without any evidence of locoregional recurrence or metastasis. We present this case in view of its aggressive nature.

3. Discussion

SC is a rare tumor with sebaceous differentiation. Although it is a slow-growing tumor, it occasionally shows rapid and aggressive behavior. It constitutes 0.2–4.6% of all malignant epithelial lesions[3]. SC predominates in the periocular region and occurs more frequently in Asian population and in women more than 40 years of age[4]. Since there are abundant sebaceous glands over the face and scalp, these areas are often affected by extraocular SC. Although SC rarely occurs in other parts of the body, it may occur in certain areas, including the trunk, extremities, genitalia, and external auditory meatus. Although aggressive behavior in SC of the scalp is rare, in our case, the tumor, which was located over the scalp, showed aggressive behavior.

The risk factors for SC include patient's weak immune system, advanced age, excess exposure to ultraviolet rays from the sun, medications, radiation, immunosuppression, inherited diseases suh as Muir-Torre syndrome[5]. Patients with Muir-Torre syndrome may have malignancy along with sebaceous tumor-like adenoma or SC. Clinically, SC presents as an asymptomatic and yellowish nodular lesion, often with ulceration. The primary sites include the eyelid (38.7%), scalp, and neck (8.7%)[6].

The pathogenesis of SC is unknown. It may begin as an inflammatory condition, which is often overlooked. The

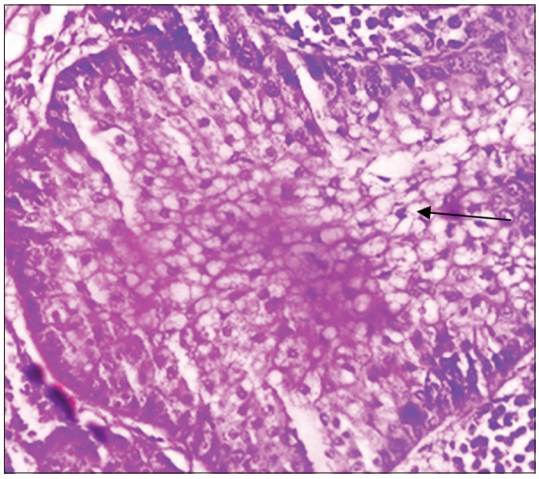

Figure 3. Photomicrograph showing neoplastic cells having hyperchromatic and pleomorphic cells with multivacuolated or foamy cytoplasm (arrow) (H&E stain, ×100).

pathogenic germline variants of DNA mismatch repair genes *MSH2, MSH6,* and *MLH1* have been identified in 8 – 29% of individuals with SC[7]. In these patients, the tumors are characterized by microsatellite instability.

On microscopy, sebaceous neoplasm shows a wide range of differentiation: Sebaceous adenoma, basal cell carcinoma with sebaceous differentiation, and sebaceous carcinoma[8]. In SC, the tumor cells are arranged in cords and lobules, the neoplastic cells show varying degrees of sebaceous differentiation, and there is tumor infiltration to adjacent soft tissue, nerve, or the lymphatic system. Less commonly, it exhibits a broad superficial intraepidermal pattern.

The architecture usually consists of sheets or lobules separated by fibrovascular stroma. The various histologic patterns of SC include lobular, cystic with the central comedo-type necrosis, papillary, and mixed type. The atypical sebocytes may be well, moderately, or poorly differentiated. They are arranged as rounded nodular aggregates or angulated infiltrative aggregates. A well-differentiated SC shows increased proportion of mature appearing multivacuolated sebocytes with nuclear indentation, mild nuclear pleomorphism, and minimal mitoses and necrosis. In contrast, anaplastic cells, with prominent nuclear pleomorphism and frequent mitoses and necrosis, are observed in moderate to poorly differentiated SC. In terms of tumor dissemination, these tumors may occasionally spread in a pagetoid manner[9]. Tumor multicentricity, differentiation, pagetoid spread, and perineural, vascular, and lymphatic invasion should be emphasized in pathological reporting since this information can aid clinicians in treating patients with SC.

Sebaceous adenoma, basal cell carcinoma with sebaceous differentiation, clear cell melanoma, clear cell squamous cell carcinoma, clear cell hidradenocarcinoma, metastatic renal cell carcinoma, and prostate carcinoma are among the benign and malignant conditions that are considered differential diagnoses for SC[10].

Sebaceous adenoma is a benign epithelial neoplasm with hyperplasia of sebaceous lobules associated with expansive aggregates of basaloid germinative cells. It is a well circumscribed neoplasm that principally demonstrates organoid and lobular configuration and contains a significant percentage of mature and lipid-rich sebaceous cells.

Another differential is basal cell carcinoma with sebaceous differentiation. Its tumor cells are small basaloid with peripheral palisading, surrounded by fibromyxoid stroma, with focal differentiation toward mature, benign-appearing, and multivacuolated sebocytes. In contrast, there is no peripheral palisading in SC.

Clear cell squamous cell carcinoma, on the other hand, shows clear cells; however, their cytoplasm is not as multivacuolated as sebocytes. Without lobular arrangement, it is difficult to determine sebaceous differentiation in squamous cell carcinoma[11].

On immunohistochemistry, SC tumor cells are reactive for epithelial membrane antigen (EMA), cytokeratin, Ber-EP4, and adipophilin (adipose differentiation-related protein, ADP), which has a membranous vesicular pattern, and androgen receptor[12]. Androgen receptors and ADPs are not observed in squamous cell carcinoma, but EMA, cancer antigen (CA)15-3, Ber-EP4, and ADP are observed in basal cell carcinoma, thus differentiating them from SC. Immunohistochemistry can also be used to evaluate *MSH2*, *MSH6*, *MLH1*, and *PMS2* for microsatellite instability in SC, as SC may metastasize in 2.4% of cases. According to the literature, local recurrence is more common in extraocular SC[12].

One of the features that is indicative of poor prognosis is tumor size >1 cm (associated with a 5-year mortality rate of 50%). The other features include moderate-to-poor sebaceous differentiation, tumor necrosis, increased mitotic activity, infiltrative growth, and lymphovascular invasion.

For local disease, wide local excision is the preferred treatment. Other modalities of treatment include Mohs micrographic surgery, radiation, and systemic chemotherapy, which may be considered for recurrent or metastatic disease[13].

Bailet *et al*. have reported a local recurrence rate of 29%, regional nodal metastasis in 15%, and a disease-related mortality of 20%[9]. It has also been reported that the 5-year survival rate of this SC is 92.7%. SC is primarily treated with wide local excision, and in cases of localized tumor, the prognosis is good following surgical removal. However, in scalp SC, adjuvant treatment with radiation and chemotherapy is required. A study conducted by Angela Orcurto *et al*. noted the recurrence of aggressive SC of the scalp even after multiple excisions and local radiotherapy[14]. In another study, Bhavaraju noted aggressive SC over the scalp and suggested the need for close follow-up of these patients to detect recurrence and distant metastasis[15]. In our case, the patient was asymptomatic without any evidence of locoregional recurrence on follow-up.

4. Conclusion

Extraocular SC is an aggressive malignant neoplasm of the skin. The management of SC is challenging and patient assessment may be necessary depending on the prognostic features. We present a case of SC of the scalp along with

clinical and histopathological findings in light of its rarity and aggressive behavior.

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The authors declare no conflicts of interest.

Author contributions

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Supervision: Sunil V. Jagtap
Investigation: Sunil V. Jagtap, Swati S. Jagtap, and Shefali Mishra
Writing – original draft: Sunil V. Jagtap and Swati S. Jagtap
Writing – review & editing: Sunil V. Jagtap, Swati S. Jagtap, Shefali Mishra, Kaushiki Varshney, and Shuchita Gaur

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ACCSCIENCE PUBLISHING

Tumor Discovery

CASE REPORT

Cystic hygroma in a young adult: A case report and recent management

Sachin S. Kadam[1]* and Tejaswini Kadam[2]

[1]Department of Surgical Oncology, Currae Cancer and Multispeciality Hospital, Mumbai, Maharashtra, India

[2]Department of Ophthalmology, Conwest and Jain Superspeciality Eye Hospital, Mumbai, Maharashtra, India

Abstract

We are reporting a case of a 27-year-old young female who presented with right side neck swelling without any associated obstructive symptoms and any other grave signs and symptoms. She noticed a gradual increase in the size of the swelling within a period of 2 years. After investigation and surgical excision, the swelling was diagnosed as cystic hygroma. The root cause of the development of cervical lymphangioma is the congenital malformation of the developing lymphatic system. Cystic hygroma is benign in nature and the cause in adults is still unclear. The most common site of origin is in head and neck region, and cystic hygroma accounts for 75% of lymphatic malformations. The most common presentation of cystic hygroma is painless swelling with ill-defined lesion, most commonly located at the posterior triangle of the neck. The common age group is between birth and 2 years of age, with very rare presentation in adults. Hence, it is necessary to rule out all differential diagnosis of cervical lymphangioma, which is presented with cystic neck swelling. Complete surgical excision is the recommended standard treatment.

Keywords: Cystic hygroma; Young adult; Cervical lymphangioma; Congenital lymphatic malformations

***Corresponding author:**
Dr. Sachin S. Kadam
(kool_sachin555@yahoo.com)

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1. Introduction

The incidence of cervical lymphangioma (cystic hygroma) in adults is very rare, and very few cases have been reported in the literature[1,2]. Lymphangiomas are congenital malformations of the developing lymphatic system, and these conditions are benign in nature. The most common cause of lymphangioma development is an obstruction or sequestration of the developing lymphatic vessels[3,4]. The cause of cystic hygroma in adults is not known; however, upper respiratory tract infection and trauma have been reported in the literature[5,6]. Diagnosis of cystic hygroma in adults is difficult, and the definitive diagnosis is purely based on the final histopathological examination. The most common location of cystic hygroma is at the posterior triangle of the neck, while the most common pathologies are inflammatory, metastatic adenopathies or lymphoproliferative diseases. Under 2 years of age, the common pathologies of cystic hygroma are branchial cysts, hemangiomas, and lymphangiomas[7]. They are characterized as slow-growing tumors, and chances of spontaneous regression are

very rare[8]. Surgical excision is the choice of treatment as described in most literature[9].

2. Case presentation

A young female of age 27 years with no co-morbidity with Eastern Cooperative Oncology Group Performance Status 1 approached our clinic and complained of right side neck swelling. There was no supportive family, medical and surgical history. The patient has a history of swelling on the right side of the neck around 2 years ago, and the swelling gradually grew (in size) over the years. There was mild pain with the movements of the neck. There was no associated history of trauma, difficulty in swallowing, and previous procedure. During clinical examination, there was mobile and fluctuating large swelling of approximately 10 cm × 9 cm in size, which was present in the right posterior triangle of the neck without neck lymphadenopathy. She had been evaluated outside of our clinic with contrast-enhanced computed tomography (CECT) of the neck and fine needle aspiration cytology (FNAC), and we also had advised her to undergo computed tomography (CT) of the chest. The finding of CECT was suggestive of a multiseptated cystic lesion of 10.5 cm × 9.5 cm × 8 cm in the posterior triangle of right side of the neck with preservation of all fat planes, as shown in Figures 1 and 2. Meanwhile, the finding of FNAC was suggestive of lymphangioma, which was probably cystic hygroma. The patient was advised to undergo surgery, and she was treated with wide local excision with intact capsule (Figures 3 and 4). The post-operative course was uneventful, and she was discharged on the 5th post-operative day. The final histopathology report confirmed that the cystic lesion was a cystic hygroma. Recurrence was not reported by the patient even after 1 year of completion of treatment.

3. Discussion

The incidence of lymphangiomas is in the range of 1.2 – 1.8/1000 of new births[10] or 1 in 2000 – 4000 live births[11], as reported in different studies. It has been found that in 90% of reported cases, the lesion occurs commonly in the age range between birth and 2 years[12]. The most common site or origin of lymphangiomas is the head and neck region[13]. The other reported sites of lymphangiomas with the lower incidence rate are retroperitoneum, axilla, pelvis, and mediastinum[14,15]. Out of all head and neck lymphatic malformations, cystic hygroma accounts for the majority with an accountability of 75% in the head and neck region[16]. The causes of lymphatic malformations are still unclear; however, some of the etiologies have been reported like misplacement of lymphatic channels during embryogenesis, arrest of lymphatic growth, and failure of lymphatic system to reach the venous drainage[17]. In addition, an association has been found between cystic hygroma and other conditions such as chromosome aneuploidies, hydrops fetalis, and intrauterine death[18].

The most common presentation of cystic hygroma is a painless and ill-defined swelling or mass. It never involves skin and it shows positive transillumination test. The common location of cystic hygroma is in the anterior and posterior triangles of the neck, with the posterior triangle of the neck being the most common site of occurrence[19]. Some of the cystic hygromas present with large neck masses with obstructive symptoms such as dysphagia or adult respiratory distress if they are located in the suprahyoid region, and these lesions are associated with a higher rate of recurrence, complications, and morbidity[20]. Incomplete surgical resection, midline location, and multiple lesions are in favor of higher recurrence rate. Hence, complete

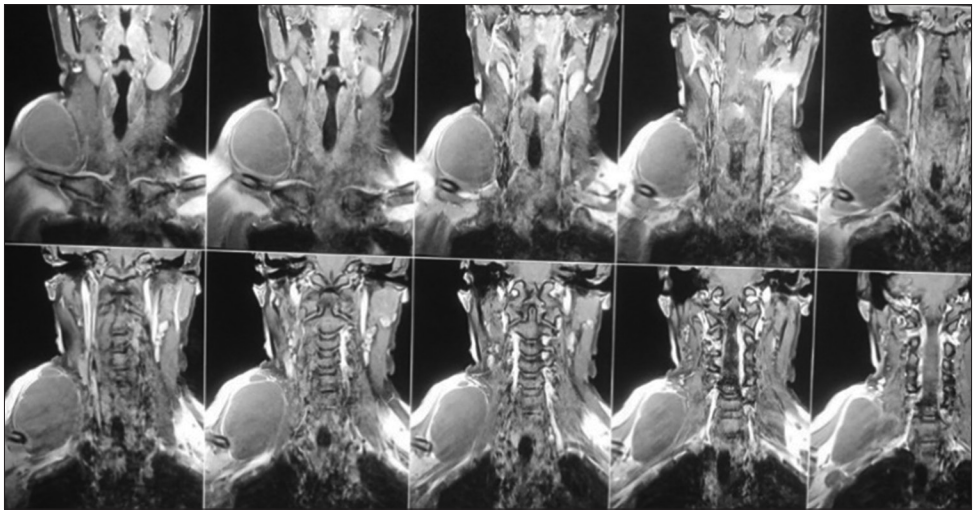

Figure 1. Coronal view of contrast-enhanced computed tomography.

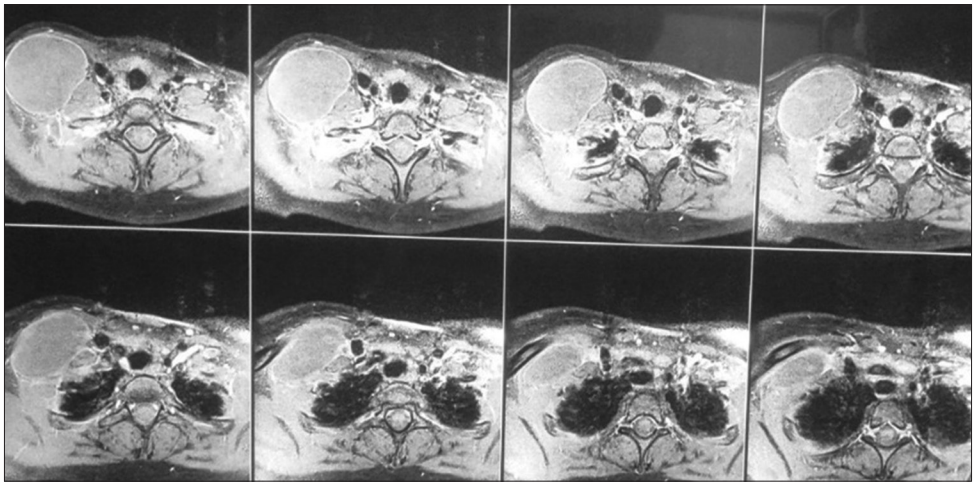

Figure 2. Axial view of contrast-enhanced computed tomography.

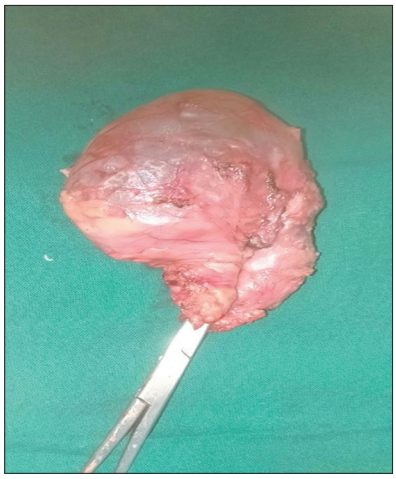

Figure 3. Specimen resected *en bloc*.

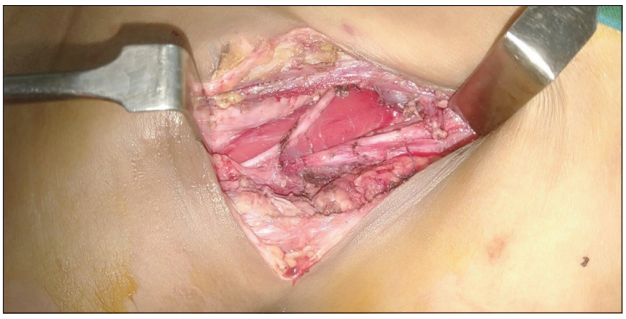

Figure 4. Post-resection.

surgical excision with intact capsule is mandatory to avoid future complications.

The choice of radiographic investigations is ultrasound, CT, and magnetic resonance imaging (MRI). CT defines the extent of the lesion with characteristics of the inner content of the cyst, while MRI helps in defining the relationship of the cystic lesion with surrounding soft tissues[21]. In most of the cases, radiographic investigations are enough for diagnostic purposes. Tissue diagnosis before surgery is only indicated if there is a dilemma in diagnosis to differentiate lymphangiomas from neck sarcoma, lymph node mass, lymphoma or other benign neck tumors. If obstructive symptoms are present like respiratory distress, prior tracheostomy is essential to maintain airway track. Classification based on anatomical location has been published in 1995 by de Serres *et al.*[22] (Table 1).

Different treatment modalities have been proposed for the treatment of cystic hygromas. In individuals with age <3 years and lesion size <4 cm, observation is a treatment option as there are chances of spontaneous regression[22,23]. The next proposed options are sclerotherapy with doxycycline or radiotherapy, which were recommended by Miceli and Stewart[24]. The other non-surgical options are percutaneous drainage, carbon dioxide laser, Nd-YAG laser, and diathermy which were proposed by Fageeh *et al.*[23] Previously sclerosing agents were used for the treatment, including boiling water, quinine, sodium morrhuate, urethane, iodine, doxycycline, and nitromin; however, sclerosing agents have been found to cause more complications with a low success rate in treatment[25-27]. Several case reports have been published establishing the role of bleomycin as primary intra-lesional sclerosing agent for the treatment of cystic hygroma[28,29]. Aspiration of cystic hygroma is one of the temporary treatment options, which helps in reducing the size of the hygroma and thereby reduces the pressure effects on the respiratory and feeding tract[30,31]. The surgical resection of the cystic hygroma is a traditionally accepted, standard treatment. However, when the lesion extends into the floor of mouth, parapharyngeal spaces or deep neck spaces, complete removal of the lesion will be a difficult task. In these

Table 1. Classification of lymphatic malformations based on anatomical location

Class	Description
Stage I	Unilateral Infrahyoid lesion
Stage II	Unilateral Suprahyoid lesion
Stage III	Unilateral Suprahyoid and Infrahyoid lesion
Stage IV	Bilateral Suprahyoid lesion
Stage V	Bilateral Suprahyoid and Infrahyoid lesion

cases, alternative procedures, such as sclerotherapy with tetracycline, bleomycin, and triamcinolone or drainage, have been recommended. The next therapeutic treatment option is radiofrequency ablation[21]. OK-432 (Picibanil), a sclerosing agent recommended by Ogita *et al.*[32], was prepared by incubating streptococcal pyogenes with penicillin[33,34]. It is used to perform sclerosis in cystic lesions of the neck as it has a property of inducing fibrosis secondary to inflammatory and cicatricle changes with the consequent contraction of the lymphangioma.

4. Conclusion

Although cystic hygroma is rare in adults, differential diagnosis among all cervical lymphangiomas is necessary. Surgical excision is the gold standard for the treatment of cystic hygroma, except in complex cases, while histopathology is the definitive diagnostic modality.

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Conflict of interest

The authors declare that they have no conflict of interests. All authors declare that they have no financial relationships at present or within the previous 3 years with any organizations that might have an interest in the submitted work and have no other relationships or activities that could appear to have influenced the submitted work.

Ethics approval and consent to participate

Informed consent was obtained from the patient for being included in this study.

Consent for publication

Informed consent to publish this case was obtained from the patient.

Availability of data

Not applicable.

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Language

All submissions must be written entirely in good American English. Spelling and use of punctuations should conform to conventions in American English. Clarity and conciseness are critical requirements for publications; therefore, submissions that are not clearly written will be returned to authors. Authors must ensure that their manuscripts are submit-ready or publish-ready before making submission. The articles published in *Tumor Discovery* are in adherence with the publishable standards of academic and scientific writing.

Please note that utilizing a language editing service is not a guarantee of acceptance.

Letter capitalization

Use sentence case capitalization in all aspects of the submission. In sentence case, most major and minor words are lowercase (proper nouns, including name of organizations and name of guidelines, are an exception in that they are always capitalized for the first letter of each word, except for minor words, such as conjunctions and short prepositions). The first letter of the first word should always be uppercase.

Manuscript title

The title should capture the conceptual significance for a broad audience. The title should not be more than 50 words and should be able to give readers an overall view of the paper's significance. Titles should avoid using uncommon jargons, abbreviations and punctuation.

Abstract

The purpose of abstract is to provide sufficient information and capture essential findings and/or messages of the paper. For full-length article, the length of an abstract should be in the range of 200-300 words. The abstract should be **unstructured**. Abstract is needed in original research article, review article, perspective article, case report and special feature article.

Keywords

Each submission should be accompanied by 3-6 keywords. Avoid using abbreviations and acronyms in keywords, unless they are established standard keywords. Separate keywords with semi-colons (i.e, term1; term2; term3).

Abbreviations and acronyms

Define abbreviations and acronyms upon their first appearance, **separately**, in the abstract, main text, table legends, and figure captions and legends.

Sections in article

(1) Section headings

Section headings should be in boldface. Examples of section headings of different levels are shown in the following:

Primary level : **1. Heart disease**

Secondary level : **1.3. Risk factors for heart disease**

Tertiary level : **1.3.2. Hypertension**

Authors are suggested **NOT** to introduce further sub-sections after the tertiary level section (e.g., **1.3.2.1. High-salt diet**).

(2) Special sectioning requirements for an original research article

- The introduction should provide a background that gives a broad readership an overall outlook of the field and the research performed. It tackles a problem and states its important regarding with the significance of the study. Introduction can conclude with a brief statement of the aim of the work and a comment about whether that aim was achieved.

- **Materials and Methods.** This section provides the general experimental design and methodologies used. The aim is to provide enough detail to for other investigators to fully replicate the results. It is also required to facilitate better understanding of the results

obtained. Protocols and procedures for new methods must be included in detail for the reproducibility of the experiments. Informed consent should be obtained from patients or parents before the experiments start and should be mentioned in this section. For human and/or research, research ethics information, such as ethics approval identifiers and the name of Institutional Ethics Review Board or Institutional Review Board, should be indicated in this section.

- This section focuses on the results and findings of the experiments performed. After (statistical) analysis, all results, including tables and figures, must be neatly presented. If necessary, this section can be sub-divided into multiple topical sub-sections.
- This section should provide the significance of the results and identify the impact of the research in a broader context. It should not be redundant or similar to the content of the results section.
- Use this section for interpretation only, and not to summarize information already presented in the text or abstract.

It is acceptable to merge both Results and Discussion as a single section.

Data and image processing

Post-acquisition processing of images, photos and figures should be kept minimum to ensure that the final figures accurately reflect the original data as it was captured and/or produced. Any alterations should be applied to the entire image. Any kind of alteration, including but not limited to brightness, contrast and color balance, has to be clearly stated in the figure legend and in Materials and Methods section. For simulated or model figures, the software used for production, editing, and/or processing should be mentioned. Presenting images in the same figure must be made apparent and should be explicitly indicated in the appropriate figure legends.

Data comparisons should only be made from comparative experiments (or data from the same experiment). Same piece of data or figure should not be used in multiple instances, unless the images/data describe different aspects of the same experiment (reasons must be stated, wherever appropriate, in this regard). If inappropriate image/data manipulation is identified after publication, the editors reserve the right to ask for the original data and, if that is not satisfactory, to issue a correction or retract the paper, as appropriate.

Unit of measurements

Use SI units.

Nomenclature of genus and species

Write in italics (e.g. *Escherichia coli*). The full genus and species names must be mentioned both in the manuscript title at the first appearance of an organism in an article. The abbreviation (e.g. *E. coli*) is allowed after first mention.

Nomenclature of genes, mutations, genotypes, and alleles

Write in italics. *Tumor Discovery* highly encourages the use the recommended names found in the gene nomenclature databases, for instance, **HUGO Gene Nomenclature Committee**.

Chemical compounds

Tumor Discovery requires authors to fulfill the requirements below while reporting and/or describing a chemical compound in articles:

Scenario	Requirements
Naming chemical compounds	Use either IUPAC conventions or common names such as cholesterol and cephalosporins
Reporting a new chemical compound	Provide the exact structure of the compound as well as sufficient data regarding the purity and identity of the compound
Reporting the use of a known chemical compound	Provide sufficient data regarding the source, purity and identity of the compound

Figures

Include all figures, including photographs, scanned images, graphs, charts and schematic diagrams, at the back of manuscript. Avoid unnecessary decorative effects (e.g., 3D graphs) and minimize image processing (e.g., changes in brightness and contrast applied uniformly for the entire figure should be avoided or minimized). All images should be set against white background.

All figures should be numbered (e.g., **Figure 1**, **Figure 2**) in boldface. Label all figures (e.g., axis, structures), and add caption (a brief title) and legend as a description of the illustration below each figure. Explain all symbols and abbreviations used. Each figure should have a brief title (also known as caption) that describes the entire figure without citing specific panels, followed by a legend, which is either the description of each panel or further description about the single image. Identify each panel with uppercase letters in parenthesis (e.g. (A), (B), (C), etc.) Figures must be cited in chronological manner in the text.

The preferred file formats for any separately submitted figure(s) are JPEG, PNG and TIFF. All figures should be of optimal resolution. Optimal resolutions preferred are 300 dots per inch (dpi) for RBG colored, 600 dpi for grayscale and 1,200 dpi for line art. Although there is no file-size limitation imposed, authors are highly encouraged to compress their figures to an ideal size without unduly affecting the legibility and resolution of figures.

If necessary, the editors may request author(s) to supply high-resolution and/or unprocessed images after submission or paper acceptance for pre-screening/review and production purposes, respectively.

Tables

Include all tables at the back of manuscript. Editable tables created using Microsoft Word are preferred. A table should be accompanied by a caption on top of it. Captions and legends (which are placed beneath table) should be concise. All tables should be numbered (e.g., **Table 1**, **Table 2**) in boldface. Explain all symbols and abbreviations used. Tables must be cited in chronological manner in the text.

Lists and math formulae

Lists and math formulae should be properly aligned and included within the main body of the manuscript. List them using Roman numerals in parenthesis (e.g. (I), (II), (III), (IV), etc.) Lists and math formulae must be cited in chronological manner in the text.

Lists and math formulae should be given in editable text and not as images. Use the solidus (/) for small fractional terms, e.g., X/Y. In principle, variables should be italicized.

Footnotes

Do not use footnotes.

In-text citations

Reference citations in the text should be numbered consecutively in superscript square brackets. Some examples:

- Negotiation research spans many disciplines[3,4].
- This result was later contradicted by Becker and Seligman[5].
- This effect has been widely studied[1–3,7].

Do not include citations in the Abstract.

Personal communications and unpublished works can only be used in the manuscript and are not to be placed in the References section. Authors are advised to limit such usage to the minimum. These should be made identifiable by stating the authors, year of personal communications or unpublished works, and the words “personal communication” or “unpublished” in parenthesis, e.g., (Smith J, 2000, unpublished).

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This section is compulsory and should be placed at the end of all manuscripts. Do not use footnotes or endnotes as a substitute for a reference list. The list of references should only include works that are cited in the text and that have been published or accepted for publication. Personal communications and unpublished works should be excluded from this section.

Authors being referenced are listed with their surname or last name followed by their initials. All references should be numbered (e.g., 1, 2, 3, and so on) and sequenced according to the order they appear as the in-text citations. References (especially journal article’s) should follow the general pattern: author(s), followed by year of publication, title of publication, abbreviated journal name in italics, volume number, issue number in parenthesis and lastly, page range or article ID. If the referred article has more than 3 authors, list only the first 3 authors and abbreviate the remaining authors as italicized “*et al*.” (meaning "and others"). Use of DOI is highly encouraged; include DOI, if available, after the page range or article ID. Examples of references for different types of publications are as follows:

(1) Journals

Journal article (print) with 1-3 authors:

Younger P, 2004, Using the internet to conduct a literature search. *Nurs Stand*, 19(6): 45–51.

Journal article (print) with more than 3 authors:

Gamelin FX, Baquet G, Berthoin S, *et al*., 2009, Effect of high intensity intermittent training on heart rate variability in prepubescent children. *Eur J Appl Physiol,* 105(1): 731–738.

Journal article (online) with 1-3 authors:

Jackson D, Firtko A, Edenborough M, 2007, Personal resilience as a strategy for surviving and thriving in the face of workplace adversity: A literature review. *J Adv Nurs*, 60(1): 1–9. http://doi.org/10.1111/j.1365-2648.2007.04412.x

Journal article (online) with more than 3 authors:

Hargreave M, Jensen A, Nielsen TSS, *et al*., 2015, Maternal use of fertility drugs and risk of cancer in children — A nationwide population-based cohort study in Denmark. *Int J Cancer*, 136(8): 1931–1939. http://doi.org/10.1002/ijc.29235

(2) Books

Book with 1-3 authors:

Schneider Z, Whitehead D, Elliott D, 2007, *Nursing and Midwifery Research: Methods and Appraisal for Evidence-based Practice*, 3rd edn, Elsevier Australia, Marrickville, NSW, 112–130.

Book with more than 3 authors

Davis M, Charles L, Curry MJ, *et al*., 2003, *Challenging Spatial Norms*, Routledge, London, 12–30.

Chapter or article in book

Knowles MS, (eds) 1986, Independent study, in *Using Learning Contracts*, Jossey-Bass, San Francisco, 89–96.

(3) Preprints

Preprint article with 1-3 authors:

Ulgen A, Gurkut O, Li W, 2019, Potential Predictive Factors for Breast Cancer Subtypes from a North Cyprus Cohort Analysis. *medRxiv*. https://doi.org/10.1101/19010181

Preprint article with more than 3 authors:

Wu S, Sun P, Li R, *et al*., 2020, Epidemiological Development of Novel Coronavirus Pneumonia in China and Its Forecast. *medRxiv*. https://doi.org/10.1101/2020.02.21.20026229

(4) Others

Proceedings of meetings and symposiums, conference papers:

Chang SS, Liaw L, Ruppenhofer J, (eds) 2000, *Proceedings of the twenty-fifth annual meeting of the Berkeley Linguistics Society, February 12–15, 1999: General session and parasession on loan word phenomena*. Berkeley Linguistics Society, Berkeley, 12–13.

Conference proceedings (from electronic database):

Wang T, Cook C, Derby B, 2009, Fabrication of a glucose biosensor by piezoelectric inkjet printing. *Proceedings of the Third International Conference on Sensor Technologies and Applications, 2009 (SENSORCOM-M'09)*, 82–85.

Online document with author names:

Este J, Warren C, Connor L, *et al*., 2008, Life in the clickstream: The future of journalism, Media Entertainment and Arts Alliance, viewed May 27, 2009, http://www.alliance.org.au/documents/ foj_report_final.pdf

Online document without author name:

Developing an argument, n.d., viewed March 30, 2009, http://web.princeton.edu/sites/writing/Writing_Center/WCWritingResources.htm

Thesis/Dissertation:

Gale L, 2000, The relationship between leadership and employee empowerment for successful total quality management, thesis, *Australasian Digital Thesis database*, University of Western Sydney, 110–130.

Standards:

Standards Australia Online, 2006, Glass in buildings: selection and installation, AS 1288-2006, amended January 31, 2008, *SAI Global database*, viewed May 19, 2009.

Government report:

National Commission of Audit, 1996, *Report to the Commonwealth Government*, Australian Government Publishing Service, Canberra.

Government report (online):

Department of Health and Ageing, 2008, Ageing and aged care in Australia, viewed November 10, 2008, http://www.health.gov.au/internet/main/publishing.nsf/Content/ageing

No author:

Guide to agricultural meteorological practices, 1981, 2nd ed, Secretariat of the World Meteorological Organization, Geneva, 10–20.

Note: When referencing an entry from a dictionary or an encyclopedia with no author there is no requirement to include the source in the reference list. In these cases, only cite the title and year of the source in-text. For an authored dictionary/encyclopedia, treat the source as an authored book.

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At the time of submission, authors must declare any (potential) conflicts or competing interests with any institutes, organizations or agencies that might influence the integrity of results or objective interpretation of their submitted works. For more information, see our **Conflict of Interest** policy.

Author contributions*

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This section should be included in original research articles, review articles and case reports. In *Tumor Discovery*, we encourage authors to use **Contributor Roles Taxonomy (CRediT)** in describing each contributor's specific contribution to the scholarly output in the Author Contributions section.

Definitions of each contributor role as per CRediT are as follows:

Contributor role	Definition
Conceptualization	Ideas; formulation or evolution of overarching research goals and aims.
Data curation	Management activities to annotate (produce metadata), scrub data and maintain research data (including software code, where it is necessary for interpreting the data itself) for initial use and later re-use.
Formal analysis	Application of statistical, mathematical, computational, or other formal techniques to analyze or synthesize study data.
Funding acquisition	Acquisition of the financial support for the project leading to this publication.
Investigation	Conducting a research and investigation process, specifically performing the experiments, or data/evidence collection.
Methodology	Development or design of methodology; creation of models.
Project administration	Management and coordination responsibility for the research activity planning and execution.
Resources	Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools.
Software	Programming, software development; designing computer programs; implementation of the computer code and supporting algorithms; testing of existing code components.
Supervision	Oversight and leadership responsibility for the research activity planning and execution, including mentorship external to the core team.
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Writing – review & editing	Preparation, creation and/or presentation of the published work by those from the original research group, specifically critical review, commentary or revision – including pre- or post-publication stages.

Authors are advised to follow *Tumor Discovery* 's preferred style of writing the Author Contributions statement. See an example below:

Conceptualization: Ali Jackson, Helen Meyer

Investigation: Ali Jackson, Tom Lewis-Hans, Han Xiang

Formal analysis: Han Xiang

Writing – original draft: Ali Jackson

Writing – review & editing: Helen Meyer, Joshua O'Brien

Supplementary files

This section is optional and contains all materials and figures that are excluded from the manuscript. These materials, figures or additional information are relevant to the manuscript but remain non-essential to readers' understanding of the manuscript's main content. All supplementary information should be submitted as a separate file during submission.

Supplementary figures and tables should be submitted in a single, separate supplementary file, and must be numbered, for example, **Figure S1** and **Table S1**. All tables must be editable (preferably created from Microsoft Word). The acceptable formats of images and illustrations used in figures are JPEG, PNG and TIFF. Citations of these items must be appropriately referenced in the manuscript in chronological manner, for instance, "Additional information can be found in **Table S1**." Note the additional letter **S** helps distinguish the normal from supplementary items.

Data set file are usually prepared using Microsoft Excel (in XLS or XLSX format).

Videos (MP4 format), with a constituent maximum size of 15 MB, can be uploaded as part of the supplementary file.

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If the editorial decision for a submission is major revision or minor revision, authors are advised to revise the manuscript (and possibly, the supplementary files) as per the review reports and resubmit the revision file, including the manuscript, title page and back matter, cover letter, and response/rebuttal letter, before the due date.

Revisions should be done on the latest version of the manuscript (or in some rare cases, edited manuscript provided by the editor) with the track change on. The revisions made should be described and/or clarified in the response/rebuttal letter; ideally, explanation about the revisions should be made clear with the help of page number and line number. If authors do not agree with reviewers' comments and suggestions, rebut their points with strong evidence and reasonable arguments.

Tumor Discovery

Special Issue Alerts

Invitation for Special Issue Proposals

Organizing and editing for a Special Issue helps Guest Editors gain editorial experience and improve academic profile, in addition to being a part of organizing scientific communication of contemporary topics.

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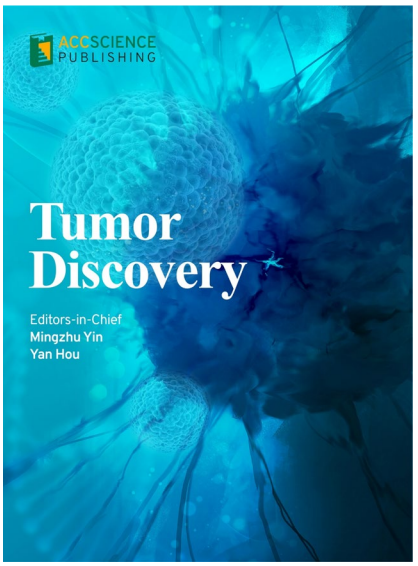

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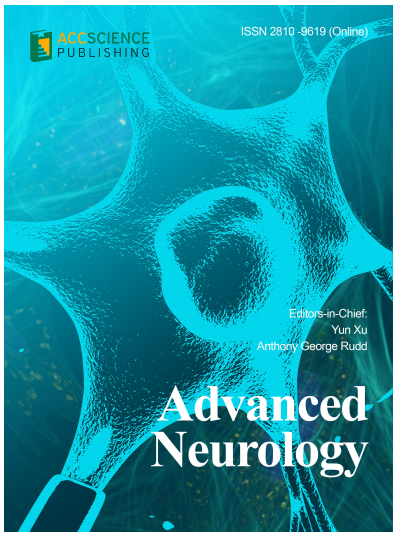

Advanced Neurology is a peer-reviewed and open-access journal that aims to publish and disseminate novel research in the breadth of neurology and neuroscience. The journal aims to advance our understanding in the nervous system and provide a platform to neuroscientists and physicians to showcase their findings in original fundamental and clinical research as well as to present new ideas that highlight the changes in the neurological clinical practice.

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