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High *MCM6* expression promotes proliferation and correlates with poor prognosis in triple-negative breast cancer

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Abstract. – OBJECTIVE: Triple-negative breast cancer (TNBC) is an aggressive subtype with a poor prognosis. Minichromosome maintenance genes (*MCM2-7*) crucial for DNA replication are significant biomarkers for various tumor types; however, their roles in TNBC remain underexplored.

MATERIALS AND METHODS: We utilized four TNBC-related GEO databases to examine *MCM2-7* gene expression and predict its prognosis in TNBC, performing single-cell analysis and GSEA to discover *MCM6*'s potential function. The Cancer Dependency Map gene effect scores and CCK8 assay were used to assess *MCM6*'s impact on TNBC cell proliferation. The correlations between *MCM6* expression, immune infiltrates, and immune cells were also analyzed. WGCNA and LASSO Cox regression built a risk score model predicting TNBC patient survival based on *MCM6*-related gene expression.

RESULTS: *MCM2-7* gene expression was higher in TNBC tissues compared to adjacent normal tissues. High *MCM6* expression correlated with shorter TNBC patient survival time. GSEA and single-cell analysis revealed a relationship between elevated *MCM6* expression and the cell cycle pathway. *MCM6* knockdown inhibited TNBC cell proliferation. A risk model featuring *MCM6*, *CDC23*, and *CCNB1* effectively predicts TNBC patient survival.

CONCLUSIONS: *MCM6* overexpression in TNBC links to a worse prognosis and reduced cell proliferation upon *MCM6* knockdown. We developed a risk score model based on *MCM6*-related genes predicting TNBC patient prognosis, potentially assisting future treatment strategies.

Key Words:
MCM2-7, TNBC, Cell cycle, Prognosis, *MCM6*.

Introduction

Breast cancer is the most commonly diagnosed cancer in women worldwide and can be divided into endocrine-dependent breast cancer, human epidermal growth factor receptor 2 (HER2)-positive breast cancer, and triple-negative breast cancer (TNBC) according to its histological characteristics[1-6]. TNBC is characterized by the lack of expression of estrogen receptor (ER), progesterone receptor (PR), and HER2 and accounts for about 10-20% of all breast cancer cases. Compared with other breast cancer subtypes, TNBC is often a highly invasive and metastatic form of breast cancer with high histological grade, presence of lymphocytic infiltration, high rate of distant metastasis, and a poorer prognosis[2,7-12]. Due to the lack of expression of PR, ER, and HER2, current EGFR-targeted therapy and hormone therapy are ineffective in treating TNBC. Chemotherapy, surgery, and radiation therapy are the most commonly used treatments for TNBC patients at present[13-15]. Despite substantial improvement in TNBC therapy, accurate prognostic biomarkers for the outcome of TNBC patients are still

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lacking so far. Therefore, there is still an urgent need to explore new prognostic biomarkers and effective therapeutic targets for TNBC[16-22].

As a well-recognized group of proteins responsible for DNA synthesis, minichromosome maintenance (MCM) has attracted more and more attention[23,24]. The family of MCM genes mainly includes 6 major genes, *MCM2*, *MCM3*, *MCM4*, *MCM5*, *MCM6*, and *MCM7*, which were evolutionally and functionally conserved[25-28]. The *MCM2-7* complex can act as DNA replicative helicase and contributes to the formation of the pre-replicative complex, which is required for DNA replication. Recent studies[29-35] suggested that the dysregulation of *MCM2-7* might act as the driving force behind tumorigenesis by disrupting genome stability and promoting cell cycle progression, both of which dramatically promote aberrant cell proliferation and increase the cancer risk. *MCM2-7* tends to exhibit abnormal expressions or activity in tumors as compared with non-tumor tissues. To date, the increased level of *MCM2-7* has been detected in various malignancies, including breast cancer, renal cell carcinoma, lung squamous cell carcinoma, gastrointestinal tract tumors, lymphomas, and brain tumors. However, there are few studies on *MCM2-7* in the field of TNBC.

The study was conducted to gain insights into the expression and prognostic significance of *MCM2-7* in TNBC. Bioinformatics analysis and functional experiments were used to determine the potential regulatory mechanism and clinical significance of *MCM6* in TNBC.

Materials and Methods

Extraction of Gene Expression and Clinical Data from TNBC Datasets

The expression of *MCM2-7* and clinical data were extracted from GEO databases. Four microarray gene expression datasets of TNBC patients, including GSE21653[36], GSE31448[37], GSE45827[38], GSE65216[39], and GSE53752, were obtained from the GEO database. The GSE21653 dataset contained 74 cases of TNBC tissues, and 29 matched adjacent normal tissues. For the remaining three datasets, 98 cases of TNBC tissues and 31 matched adjacent normal tissues in the GSE31448 dataset, 45 cases of TNBC tissues and 11 matched adjacent normal tissues in the GSE45827 dataset, 55 cases of TNBC tissues and 11 matched adjacent normal tissues in the GSE65216 dataset. The method for extracting microarray gene expression values is based on our previous research[40-42]. First, a background correction was performed with the Limma package of R (Bioconductor, Roswell Park Comprehensive Cancer Center, NY, USA). The probe ID was converted into a gene symbol. When a gene was mapped to different probes, the genic expression value was calculated by the average expression value. Next, the median normalization was performed using the robust multichip averaging method. COMBAT algorithm[43] was performed to remove the batch effect on GSE21653, GSE31448, GSE45827, and GSE65216. GSE53752 was used for the validation cohort.

Survival Analysis

Kaplan-Meier survival curve was used to estimate disease-free survival (DFS) using the GSE21653 dataset. A log-rank test was used to compare the survival distribution among different groups. Nomogram was built by Hiplot (available at: https://hiplot.com.cn/).

CancerSEA Analysis

CancerSEA is a website that contains 14 functional states (including cell cycle, EMT, proliferation, stemness, invasion, differentiation, DNA repair, apoptosis, DNA damage, metastasis, hypoxia, inflammation, angiogenesis, and quiescence) at the single-cell level (available at: http://biocc.hrbmu.edu.cn/CancerSEA/)[44]. CancerSEA contained 25 different kinds of cancer, including nearly 42,000 individual cells. In the research, CancerSEA was used to evaluate the correlation between *MCM6* expression and the functional state of breast cancer cells.

The Cancer Dependency Map

The Cancer Dependency Map is a website used to identify genes critical for the survival and proliferation of tumor cells (available at: https://depmap.org/portal/)[45-47]. A negative score for CERES indicates that the knockout gene inhibits tumor cell survival and proliferation, while a positive score indicates that the knockout gene promotes survival and proliferation. A CERES score <-1 was defined as an essential gene for tumor cell survival. CERES scores for *MCM6* in 23 TNBC cell lines were calculated.

Cell Culture and Transfection

The TNBC cell lines MDA-MB-231 and BT-549 were obtained from ATCC. MDA-MB-231

cells and BT-549 cells were respectively cultured in L-15 (Biological Industries, Israel) medium and DMEM (Biological Industries, Israel) medium. All mediums contained 100 U/mL penicillin and 0.1 mg/mL streptomycin (BBI Life Sciences, Shanghai, China) and 10% fetal bovine serum (Biological Industries, Beit HaEmek, Israel). Cells were cultivated at 37°C in the presence of 5% CO_2. Cell transfection was performed using Lip2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Patients and Specimens

Twenty TNBC patients from the patients of Fujian Provincial Hospital between August 2021 and December 2022 were identified. The study was performed with the approval of the Ethics Committee of Fujian Provincial Hospital and complied with the Helsinki Declaration (No.: K2021-04-069). The written informed consent was obtained from all participating TNBC patients. The characteristics of 20 TNBC patients are presented in Table I.

RNA Isolation and RT-PCR

TRIzol was performed to extract total RNA. Then, RNA was reverse transcribed with an mRNA reverse transcription kit (Takara, Japan). RT-PCR was performed using the SYBR Green kit (Vazyme, China). The primer sequences were shown as follows: *MCM6* forward primer 5'-TGTCAGTGGTGTTGATGGATATG-3', *MCM6* reverse primer 5'- GCTGTCTGTTCCTCATCTCTG-3'; *CCNB1* forward primer 5'-TCTGGATAATGGTGAATGGACA-3', *CCNB1* reverse primer 5'-CGATGTGGCATACTTGTTCTTG-3'; *CDC23* forward primer 5'-CTGGCCAAGGCCTACTTTGA-3', *CDC23* reverse primer 5'- TTCCAGGGGGCCTAAGCTAT-3'; *GAPDH* forward primer 5'-GGAAGGACTCATGACCACAGTCC-3'; *GAPDH* reverse primer 5'-TCGCTGTTGAAGTCAGAGGAGACC-3'. *GAPDH* was used as the loading control. Genes expression levels were determined by $2^{-\Delta\Delta CT}$ method.

Table I. Characteristics of triple-negative breast cancer patients.

Characteristics	All patients (n=20)
Age at diagnosis (years)	
≤ 50	8
> 50	12
Tumor size	
≤ 2 cm	10
> 2 cm	10
Lymph node metastasis	
No	12
Yes	8
Tumor grade	
I+II	9
III	11
Lymphovascular invasion	
Yes	13
No	7

Cell Proliferation Assays

The transfected MDA-MB-231 and BT-549 were seeded at a density of $5x10^3$ cells/well and cultured for 24, 48, or 72 h. Cell proliferation was subsequently detected using the Cell Counting Kit-8 (CCK-8) assay.

Immune Infiltration Analysis

The immune cell abundance and tumor microenvironment score were calculated by using CIBERSORT[48] and ESTIMATE[49] through Sangerbox (available at: http://sangerbox.com/Tool), respectively. CIBERSORT is a deconvolution algorithm that can estimate the cell composition of complex tissues according to standardized gene expression values. CIBERSORT could sensitively and specifically quantify the abundance of 22 immune cell types at once. ESTIMATE is an algorithmic tool used to evaluate the tumor microenvironment for each tumor sample, including stromal score, immune score, ESTIMATE score, and tumor purity.

Weighted Gene Correlation Network Analysis (WGCNA)

WGCNA is a common algorithm for building gene co-expression networks and is performed *via* the WGCNA R package through Sangerbox (available at: http://sangerbox.com/Tool). The soft threshold β was a weighted parameter of the adjacent function, which the power value was obtained by the pickSoftThreshold function in the R package WGCNA. The resulting Pearson correlation matrix was transformed into a matrix of connection strength (an adjacency matrix) using the power function [(1 + correlation)/2X soft threshold power], which was then converted to a topological overlap matrix. Signed correlation networks were used, and the power for soft-thresholding was selected according to the scale-free topology criterion. The minimum module size was set to 50 genes, and the parameter merge cut height was set at 0.25 to merge highly correlated modules[50].

Gene Ontology (GO) and KEGG Pathway Enrichment Analysis

GO biological processes (BP) and KEGG pathway enrichment were calculated by the functional enrichment tool DAVID (available at: https://david.ncifcrf.gov/)[51]. DAVID bioinformatics resources provide an integrated biological database and a repository of analytic tools for systematic exploration of the biological meaning of gene sets DAVID. The default parameters in the tool were used, and enriched pathways were ranked according to their enrichment scores. A *p*-value lower than 0.05 was identified as an enriched function.

Statistical Analysis

The results are expressed as mean±standard deviation (SD). Pearson's correlation coefficient was calculated to test the statistical correlation. A *p*-value lower than 0.05 was considered significant.

Results

The Expression and the Prognostic Significance of MCM2-7 in TNBC Patients

To explore the expression of *MCM2-7* in TNBC patients, four related GEO databases (GSE31448, GSE65216, GSE21653, and GSE45827) were employed (Figure 1). To minimize the potential batch effects for four independent databases, batch effect removal strategies were performed by the COMBAT algorithm. GSE31448, GSE65216, GSE21653, and GSE45827 were merged to remove batch effects and renamed as 4-TNBC-GEOs. From the boxplot, we can observe that the sample distribution of each dataset before removing the batch effect is different, suggesting that there is a batch effect. After removing batch effects, the data distributions between each dataset tend to be consistent (Figure 1A). From the UMAP plot, we can observe that the samples of each dataset are clustered together before removing the batch effect, suggesting that there is a batch effect. After removing the batch effect, the samples in each dataset are clustered and intertwined with each other, suggesting that the batch effect is better removed (Figure 1B). Based on 4-TNBC-GEOs, we observed that the expression of *MCM2, MCM3, MCM4, MCM5, MCM6*, and *MCM7* were significantly higher expressed in TNBC tissues compared to non-tumor tissue.

To explore the significance of *MCM2-7* in clinical prognosis, we used Kaplan-Meier survival analysis to prepare disease-free survival (DFS) curves for TNBC patients. Of 4-TNBC-GEOs, only GSE21653 has complete survival information; thus, it was further used for survival curve plots. Survival data were obtained for 74 TNBC patients in the GSE21653 dataset. The Kaplan-Meier survival analysis revealed that TNBC patients with high expression of *MCM6* exhibited shorter survival time, while the expression of *MCM2, MCM3, MCM4, MCM5,* and *MCM7* was not related to TNBC patients' survival (Figure 2).

The Role of MCM6 in TNBC Cells

Among *MCM2-7*, only *MCM6* was significantly related to the prognosis of TNBC patients. To explore the function of *MCM6* in TNBC. First, we queried the relationship between *MCM6* gene expression and 14 functional states using single-cell data from the CancerSEA database. According to data from Braune EB (cell number=369), *MCM6* gene expression was significantly positively correlated with cell cycle and DNA repair in breast cancer (Figure 3A). Next, GSEA was performed to identify the differentially activated signaling pathways in the high *MCM6* expression for 4-TNBC-GEOs data. The GSEA plot showed that high *MCM6* expression positively correlated with cell cycle and nucleotide excision repair (Figure 3B). These imply that *MCM6* may mainly regulate the cell cycle and DNA repair biological process in breast cancer cells. DepMap is a website used to identify genes critical for the survival and proliferation of tumor cells. A negative score for CERES indicates that the knockout gene inhibits tumor cell survival and proliferation, while a positive score indicates that the knockout gene promotes survival and proliferation. A CERES score <-1 was defined as an essential gene for tumor cell survival. From the DepMap website, we obtained the CERES scores of 23 TNBC cell lines. The CERES scores of *MCM6* were all less than -1 in the above cells, and the average CERES score was -1.5691 (Figure 3C). These results suggested that MCM6 may affect the proliferation of TNBC cells. Using RT-PCR, we found that *MCM6* mRNA expression was significantly higher in BT-549 and MDA-MB-231 TNBC cell lines than in MCF-10A normal breast cell lines (Figure 3D). *MCM6*-targeting shRNA was transfected into BT-549 and MDA-MB-231 cells, and MCM6 was successfully knocked down (Figure 3 E-F).

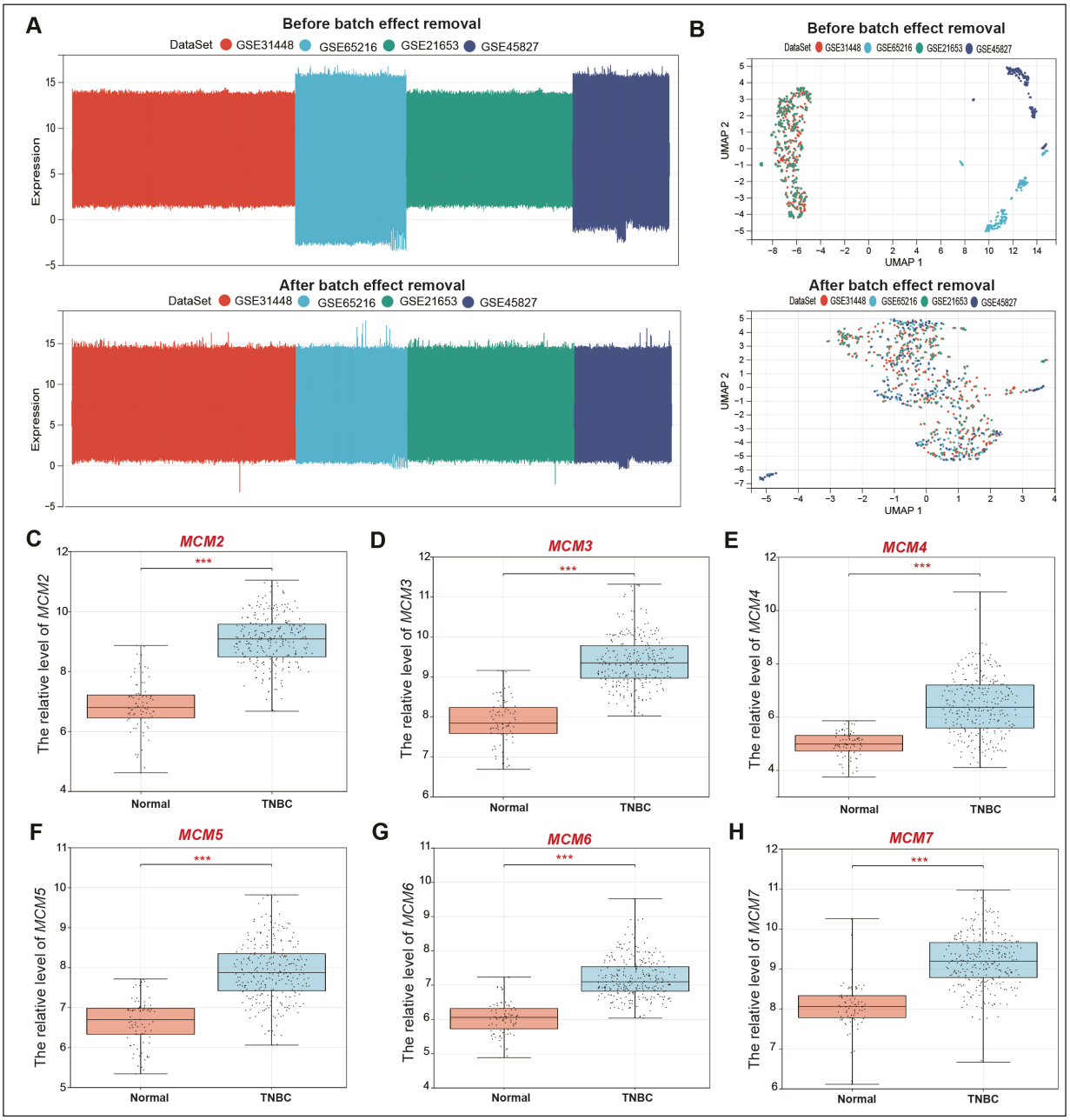

Figure 1. The expression of *MCM2-7* in TNBC patients. **A**, Boxplots of data distribution before and after batch effect removal for GSE31448, GSE65216, GSE21653 and GSE45827 datasets. **B**, Cluster diagram of samples before and after batch effect removal for GSE31448, GSE65216, GSE21653 and GSE45827 datasets. **C-H**, The expression of (**C**) *MCM2*, (**D**) *MCM3*, (**E**) *MCM4*, (**F**) *MCM5*, (**G**) *MCM6* and (**H**) *MCM7* in 4-TNBC-GEOs. *** $p < 0.001$.

CCK-8 analysis showed that *MCM6* knockdown strongly inhibited the proliferation of both TNBC cell lines (Figure 3 G-H).

The Relationship Between MCM6 Expression and Immune Infiltrates in TNBC

There is increasing evidence that the immune system is closely related to tumor development and progression. Therefore, we investigated the relationship between *MCM6* expression and immunity. Using the CIBERSORT algorithm, we evaluated the possible correlation between 22 subpopulations of infiltrating immune cells and *MCM6* expression based on 4-TNBC-GEOs data, and the heatmap showed that no infiltrating immune cells were significantly

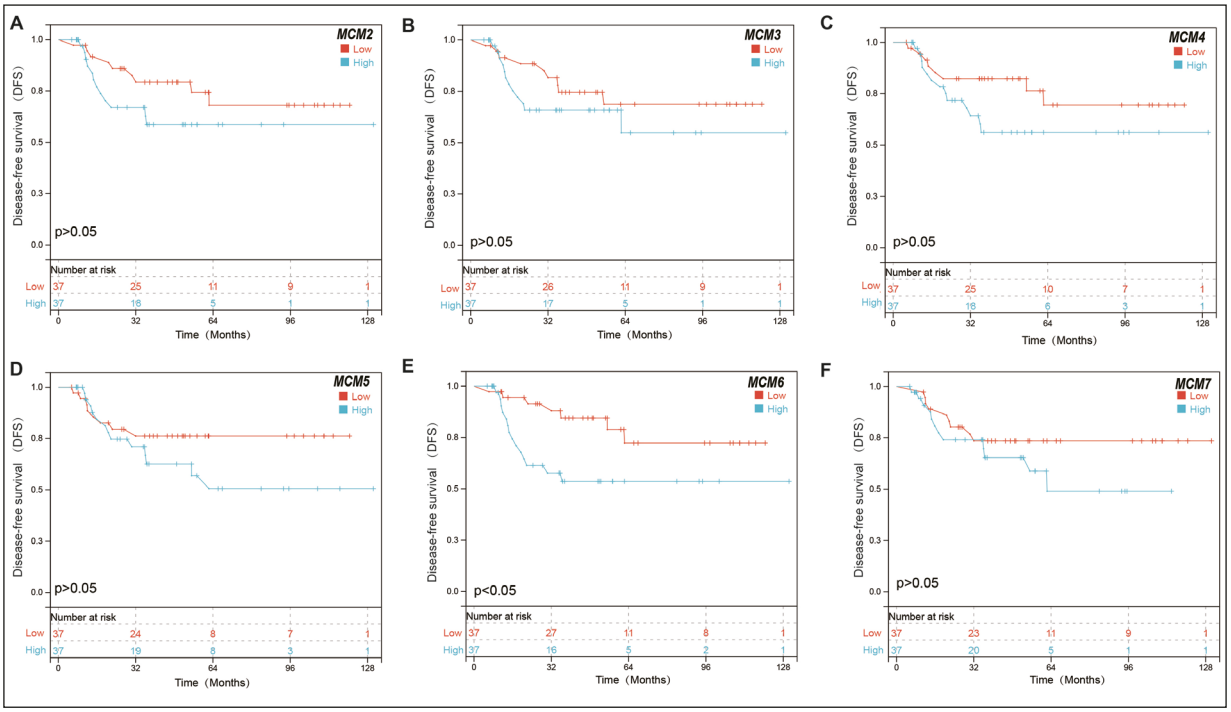

Figure 2. The DFS of *MCM2-7* for TNBC patients. Overall analysis for the prognostic value of (**A**) *MCM2*, (**B**) *MCM3*, (**C**) *MCM4*, (**D**) *MCM5*, (**E**) *MCM6* and (**F**) *MCM10* expression for DFS in TNBC patients by Kaplan-Meier analysis based on GSE21653 dataset. The Kaplan-Meier method was used to draw survival curves, and the log-rank test was performed to evaluate survival difference.

correlated to MCM6 (Figure 4A). In addition, the ESTIMATE algorithm was used to estimate stromal score, immune score, and tumor purity based on 4-TNBC-GEOs data. The results showed that TNBC patients with a higher level of *MCM6* expression specifically had a lower immune cell infiltration and a higher tumor purity (Figure 4 B-C).

Construction of Weighted Gene Co-Expression Networks for MCM6

Here, WGCNA was used to reveal the highly correlated genes and co-expression networks of *MCM6* in TNBC patients. A total of 272 TNBC samples obtained from 4-TNBC-GEOs data were used to build WGCNA. We calculated the network topology for soft-thresholding powers from 1 to 30 to choose the best threshold. The power value was the most critical parameter affecting the average connectivity degree and the independence of each co-expression module. As shown in Figure 5A, the power value 3.085 was the lowest power for the scale-free topology. Additionally, soft power showed a higher average connectivity degree (Figure 5B).

The co-expression similarity matrix was then transformed into the adjacency matrix by choosing 3.085 as a soft threshold, and a topological overlap matrix (TOM) was subsequently computed. Using the dynamic tree cut method, we obtained a total of 28 meaningful modules marked with different colors, among which the genes co-expressed with *MCM6* belong to the brown module containing 1,610 genes (Figure 5 C-D). To obtain further insight into the function of 1,610 genes in the brown module, GO and KEGG pathway analyses were conducted using the DAVID database.

The top 10 terms of KEGG pathway terms were cell cycle, RNA transport, Human T-cell leukemia virus 1 infection, cellular senescence, ribosome biogenesis in eukaryotes, DNA replication, RNA degradation, p53 signaling pathway, nucleotide excision repair, and base excision repair (Figure 5E). The top 10 terms of biological processes (BP) were cell cycle, cell cycle process, mitotic cell cycle, chromosome organization, regulation of cell cycle, regulation of cell cycle process, cell cycle phase transition, cell division, chromosome segregation, and mitotic nuclear division (Figure 5F).

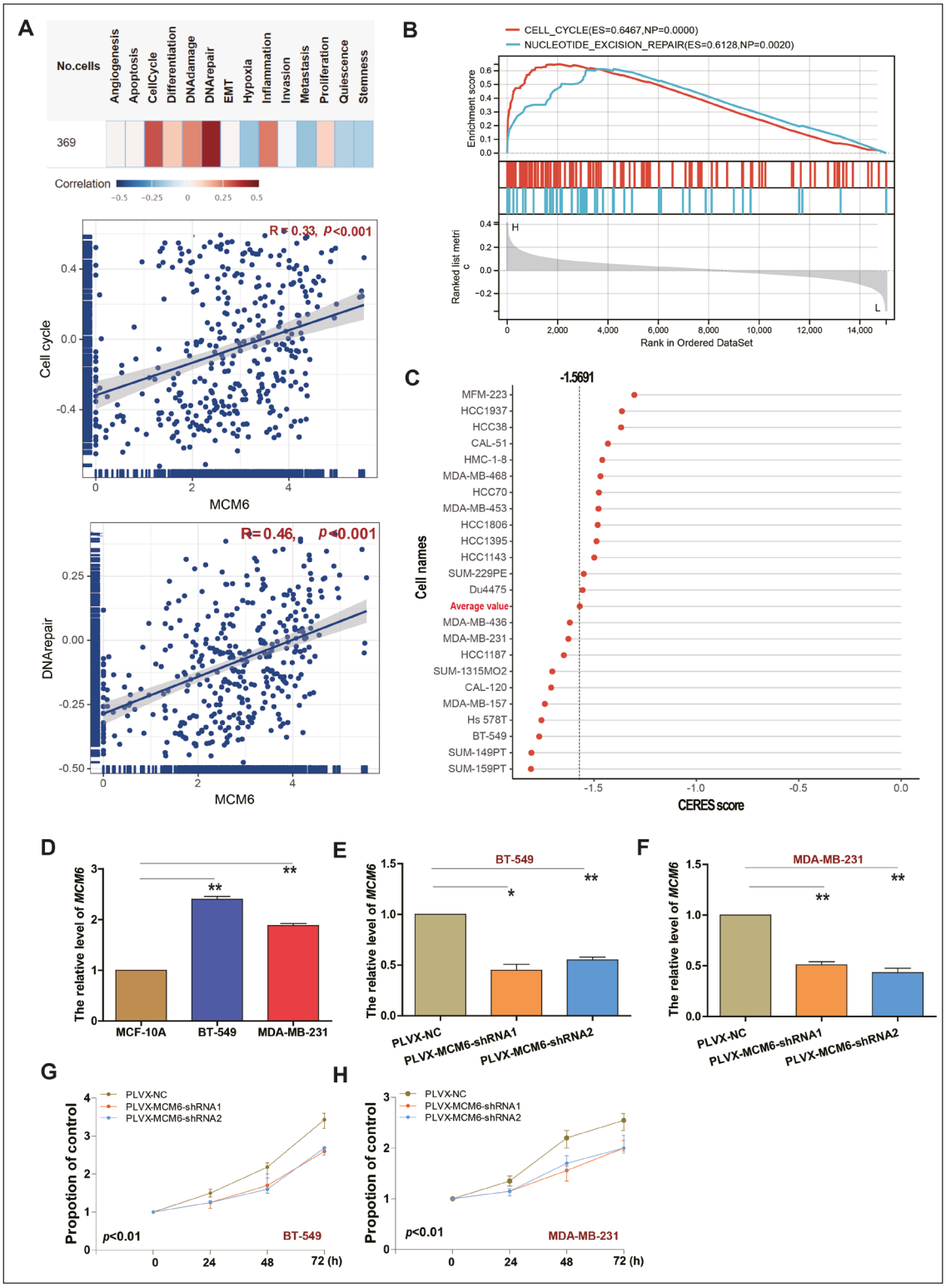

Figure 3. The function of *MCM6* in TNBC cells. **A**, Single-cell analysis indicated that MCM6 is primarily involved in regulation of the cell cycle and DNA repair in breast cancer. **B**, GSEA analysis suggested that high expression of *MCM6* is related to cell cycle and nucleotide excision repair in TNBC using 4-TNBC-GEOs datasets. **C**, The CERES score of *MCM6* in 23 TNBC cell lines. **D**, The expression of *MCM6* in MCF-10A, BT-549 and MDA-MB-231 cells. **E-F**, The expression of *MCM6* in *MCM6* knockdown BT-549 cells (**E**) and *MCM6* knockdown MDA-MB-231 cells (**F**). **G-H**, Knockdown *MCM6* inhibited the proliferation of (**G**) BT-549 and (**H**) MDA-MB-231 cells detected by CCK8. * $p < 0.05$; ** $p < 0.01$.

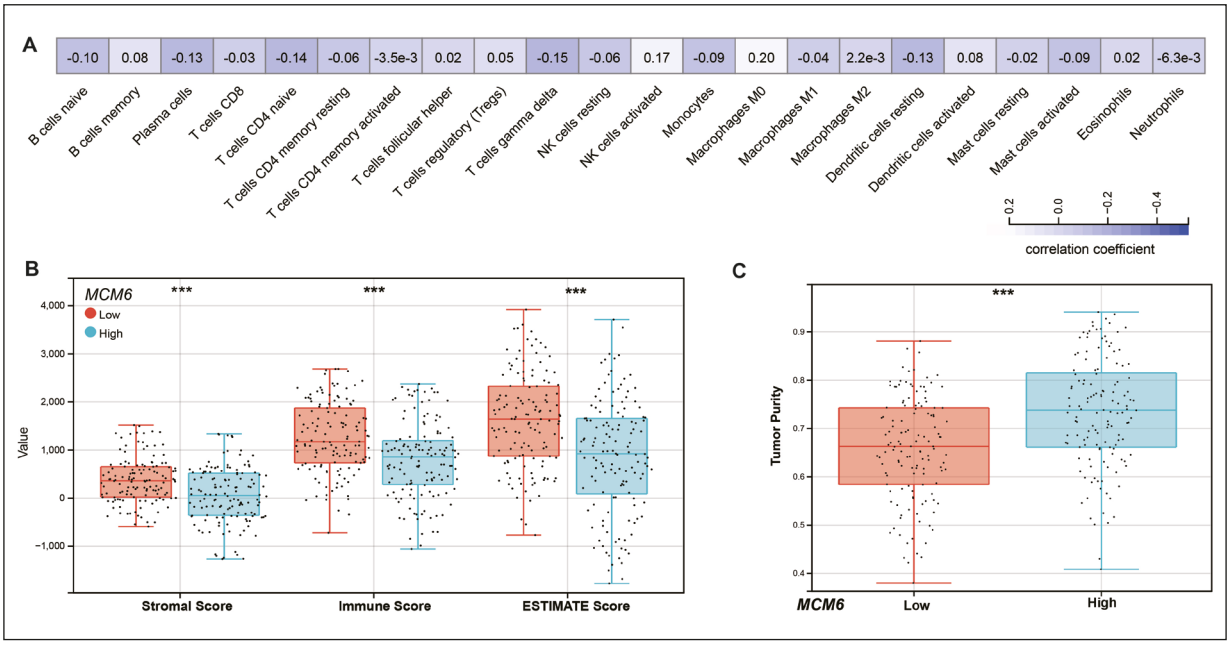

Figure 4. Correlations between *MCM6* expression and immune infiltrates in TNBC. **A**, Correlation between 22 subpopulations of infiltrating immune cells and *MCM6* expression based on 4-TNBC-GEOs data. **B**, The stromal score, immune score and estimate score in *MCM6* low and high expression groups based on 4-TNBC-GEOs data. **C**, The tumor purity score in *MCM6* low and high expression groups based on 4-TNBC-GEOs data. *** $p < 0.001$.

Prognosis Model of MCM6-Related Genes Constructed and Survival Analysis

Among the pathways identified from the KEGG and GO-BP analysis, the cell cycle pathway possessed the greatest number of gene counts, which contains 41 genes, including *MCM6*. Thus, we renamed these genes as *MCM6*-related genes. We next analyzed the prognostic value of these *MCM6*-related genes in TNBC patients based on GSE21653. The results showed that high expression of *MCM6, CDC23* (cell division cycle 23), *CCNB1* (cyclin B1), *PTTG1* (pituitary tumor-transforming 1), *PCNA* (proliferating cell nuclear antigen), *CHEK1* (checkpoint kinase 1), *CCNE2* (cyclin E2) and *PLK1* (polo-like kinase 1) were poor prognostic factors in TNBC patients (Figure 6A). As a result, these eight genes were subsequently analyzed by the LASSO regression analysis to build a prognostic model (Figure 6B). The formula of risk score was as follows: risk score = (the expression of *CDC23* × 0.66 + the expression of *CCNB1* × 0.04 + the expression of *MCM6* × 0.24) (Figure 6C). According to the risk score, the TNBC patients were divided into low and high-risk groups. Survival analysis revealed that TNBC patients in the high-risk group have a significantly worse prognosis than those in the low-risk group (Figure 6D). The distribution of risk score, survival status, and three gene expression levels of TNBC patients is shown in Figure 6E. The AUC values for 1-year, 3-year, and 5-year DFS were 0.73, 0.83, and 0.85, respectively (Figure 6F). To further verify the validity of the prognostic model, we downloaded 51 samples with complete clinical information from the GSE53752 database. Each patient was brought into the previous prognostic model to calculate the risk score. Patients were divided into high-risk and low-risk groups. Kaplan-Meier curve analysis showed that TNBC patients with low-risk scores had a better OS than those in the high-risk-score group (Figure 7).

Construction of a Clinical Prognostic Prediction Model

Based on four variables including risk score, age, stage, and grade, a nomogram was developed to calculate the probability of survival at 1-year, 3-year, and 5-year (Figure 8A). The AUC of the nomogram for 1-year, 3-year, and 5-year DFS were 0.81, 0.86, and 0.84, respectively (Figure 8B). Compared with the ideal model, the calibration curves showed our DFS nomogram to perform well (Figure 8C).

The Expression of MCM6-Related Genes in TNBC Patients

Finally, we measured *CDC23, CCNB1* and *MCM6* mRNA levels in 20 clinical TNBC sam-

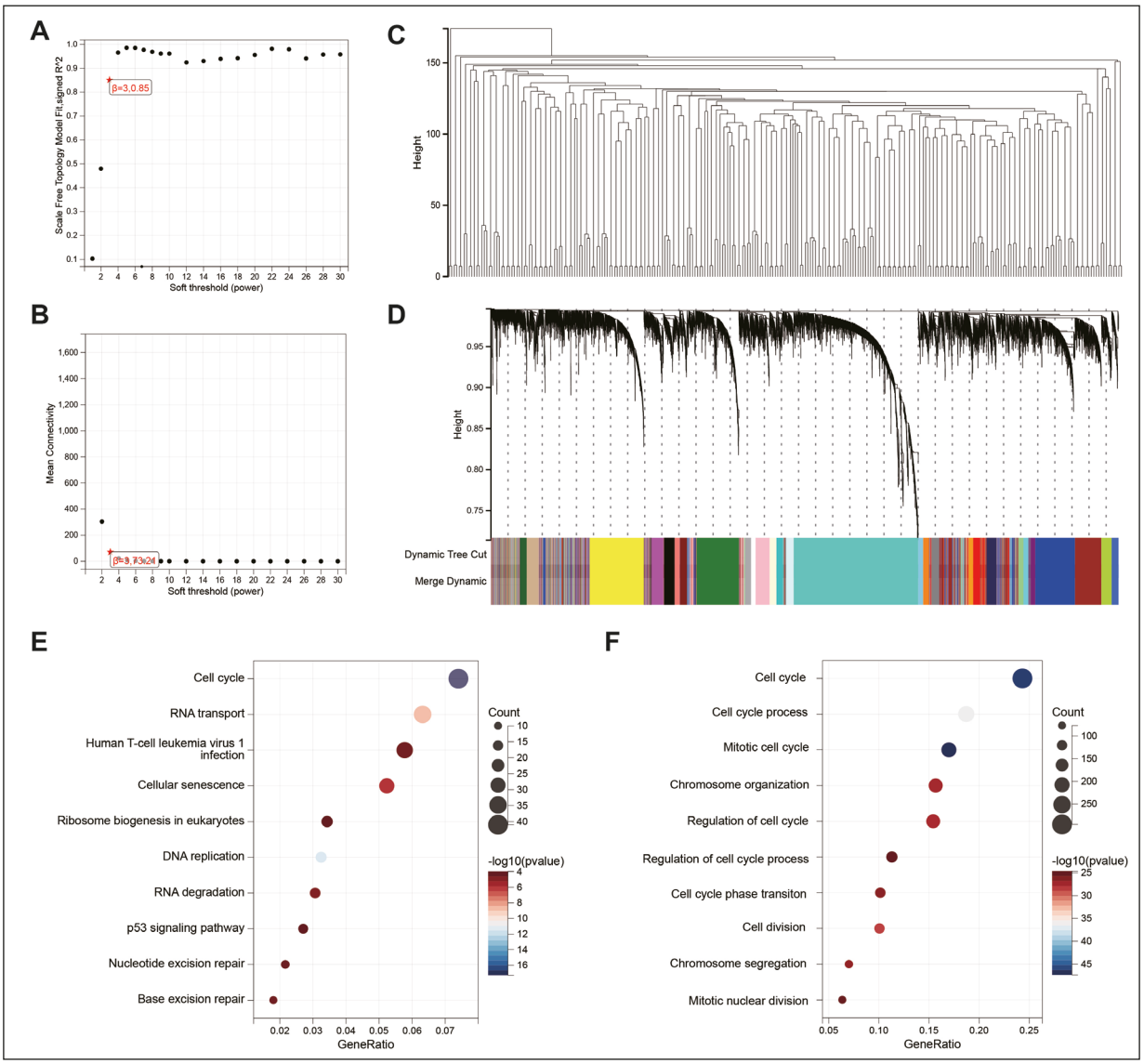

Figure 5. Identification of co-expression module genes associated with *MCM6* using the WGCNA. **A**, Relationship between scale-free topology model fit and soft-thresholds (powers) in 4-TNBC-GEOs. **B**, Relationship between the mean connectivity and various soft-thresholds in 4-TNBC-GEOs. **C**, The hierarchical clustering tree of WGCNA. **D**, Dendrogram of modules identified by WGCNA in 4-TNBC-GEOs. **E**, Results of KEGG analysis of brown model genes. **F**, Results of GO-BP analysis of brown model genes.

ples, using quantitative RT-PCR. The results showed that the expressions of *CDC23, CCNB1* and *MCM6* in TNBC tissues was significantly higher than those in adjacent tissues (Figure 9).

Discussion

MCMs family is a core component of DNA replication and is involved in the cell cycle process. Compared with non-tumor tissue, the dysregulation and dysfunctionality of *MCM2-7* are frequently observed in numerous types of cancers. There is growing evidence suggesting that aberrant *MCM2-7* promotes tumor initiation and progression by regulating genomic instability and accelerating cell proliferation.

MCM2 is not only an important component of DNA replication initiation but also plays an important role in tumor proliferation, invasion, and metastasis and is a prognostic indicator for many malignant tumors. Studies[32] have shown that *MCM2* is highly expressed in malignant tumors and is associated with higher tumor

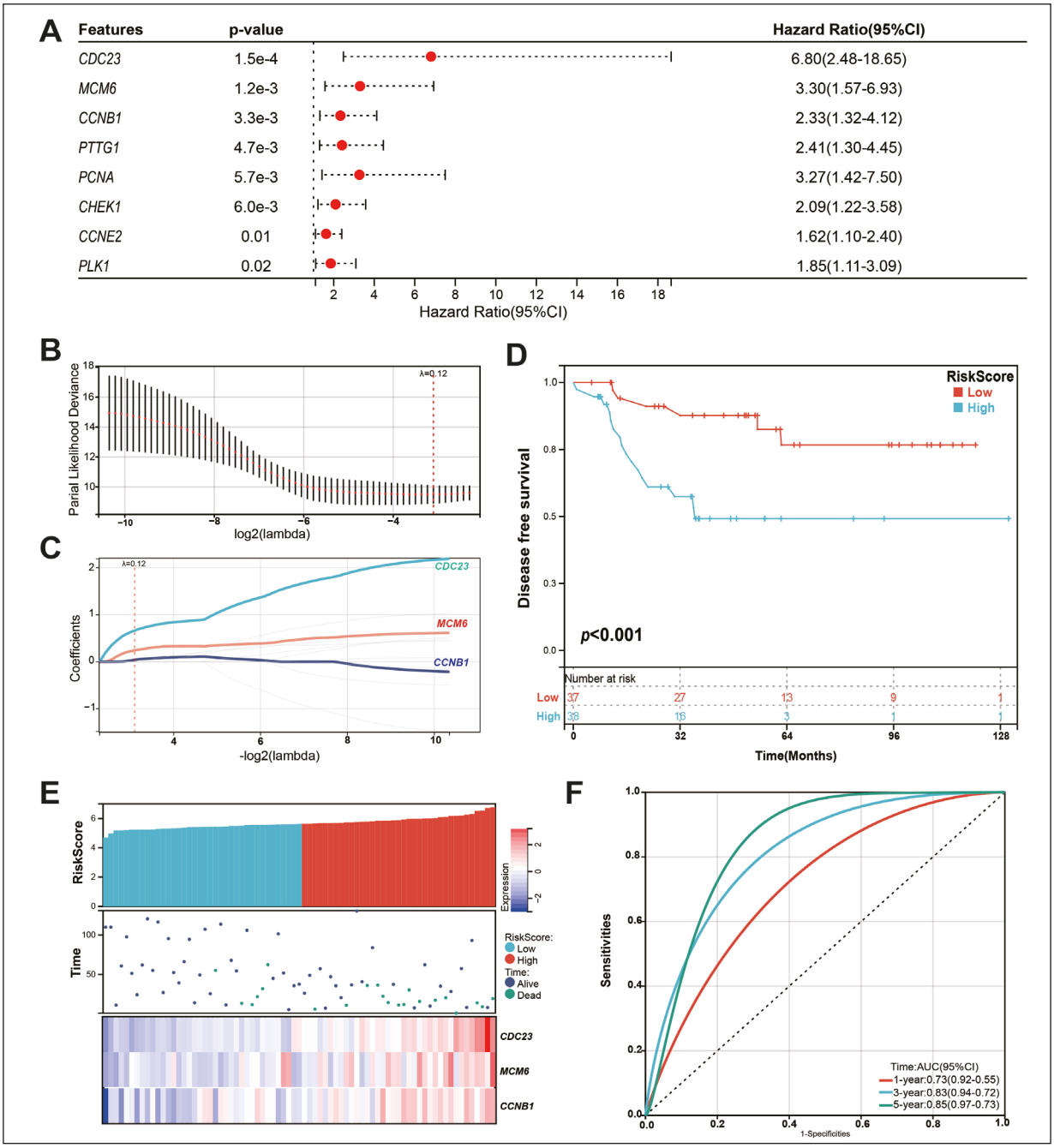

Figure 6. Construction of *MCM6*-related genes-based classifier to predict prognosis in TNBC patients. **A**, Eight *MCM6*-related genes with statistical significance in univariate Cox analysis are highlighted with detailed outputs. **B**, Partial likelihood deviance of DFS for the LASSO coefficient profiles. **C**, LASSO coefficient profiles of *MCM6*-related genes for DFS. **D**, Kaplan-Meier curves to compare DFS of low-risk and high-risk groups. (E) The distribution of risk score, survival status, and mRNA expression levels of TNBC patients in GSE21653 cohort. **F**, ROC curve of the *MCM6*-based risk score.

grades and a worse prognosis. *MCM2* is highly expressed in gallbladder cancer and lung cancer with poor differentiation, larger tumor size, higher TNM stage, and lymph node metastasis. In colorectal cancer, *MCM2* is associated with tumor pathological stage, vascular invasion, and Dukes stage. Compared with Ki-67, *MCM2* can more accurately predict the risk of bladder cancer recurrence.

Previous studies[52-55] have shown that *MCM3* is overexpressed in many tumors. *MCM3* is highly expressed in colorectal cancer and is associated

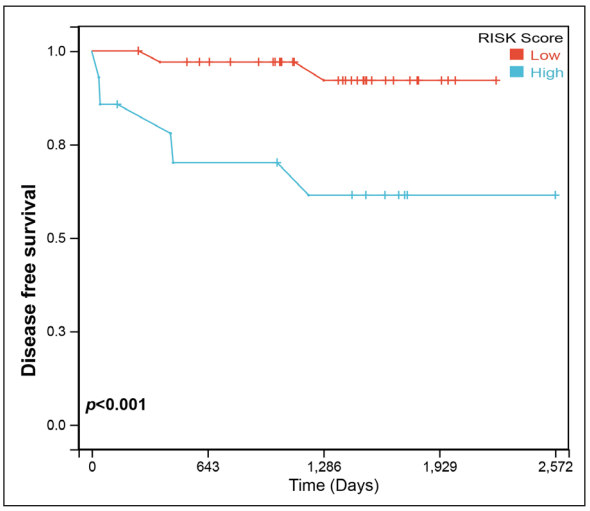

Figure 7. Validation of the risk score model using the GSE53752 database. Kaplan-Meier plot of the risk score model by using the GSE53752 database.

with poor prognosis. In addition, the knockdown of *MCM3* expression can inhibit the proliferation, invasion, and migration of colorectal cancer cells. In ovarian cancer, high expression of *MCM3* is associated with tumor malignant progression. Compared with Ki67, *MCM3* is a better prognostic marker in patients with invasive ductal breast cancer[55].

MCM4 has been reported to be a marker that differentiates malignant melanocyte skin lesions and is associated with shorter survival in melanoma patients[56]. High expression of *MCM4* was significantly associated with shorter survival in esophageal adenocarcinoma[57]. In breast cancer, a high level of *MCM4* expression is associated with disease progression, high-grade breast tumors, and shorter survival[58].

Increased expression of *MCM5* has been found in several types of cancer, including oral squamous cell carcinoma, lung cancer, gastric adenocarcinoma, kidney cancer, cervical cancer, and bladder cancer. In oral squamous cell carcinoma, high expression of *MCM5* is associated with malignant progression and poor prognosis[58]. *MCM5* is highly expressed in lung cancer and is associated with a shorter survival time in patients with lung adenocarcinoma. *MCM5* can promote the

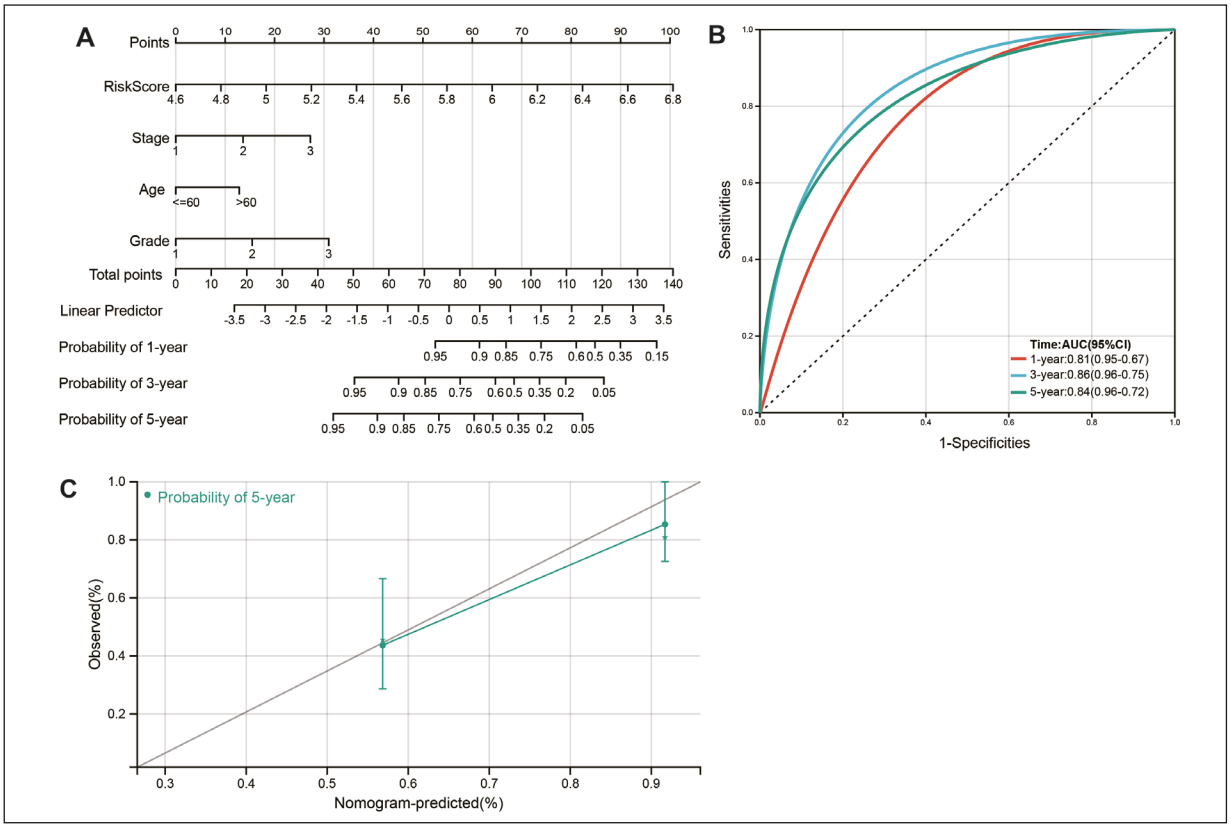

Figure 8. Nomogram and calibration plots for the prediction of outcomes in TNBC patients based on risk score. **A**, Nomogram for predicting 1-year, 3-year and 5-year events that combine clinical data with age, stage, grade, and risk score. **B**, ROC curves of the combined nomogram. **C**, The calibration plots for predicting DFS.

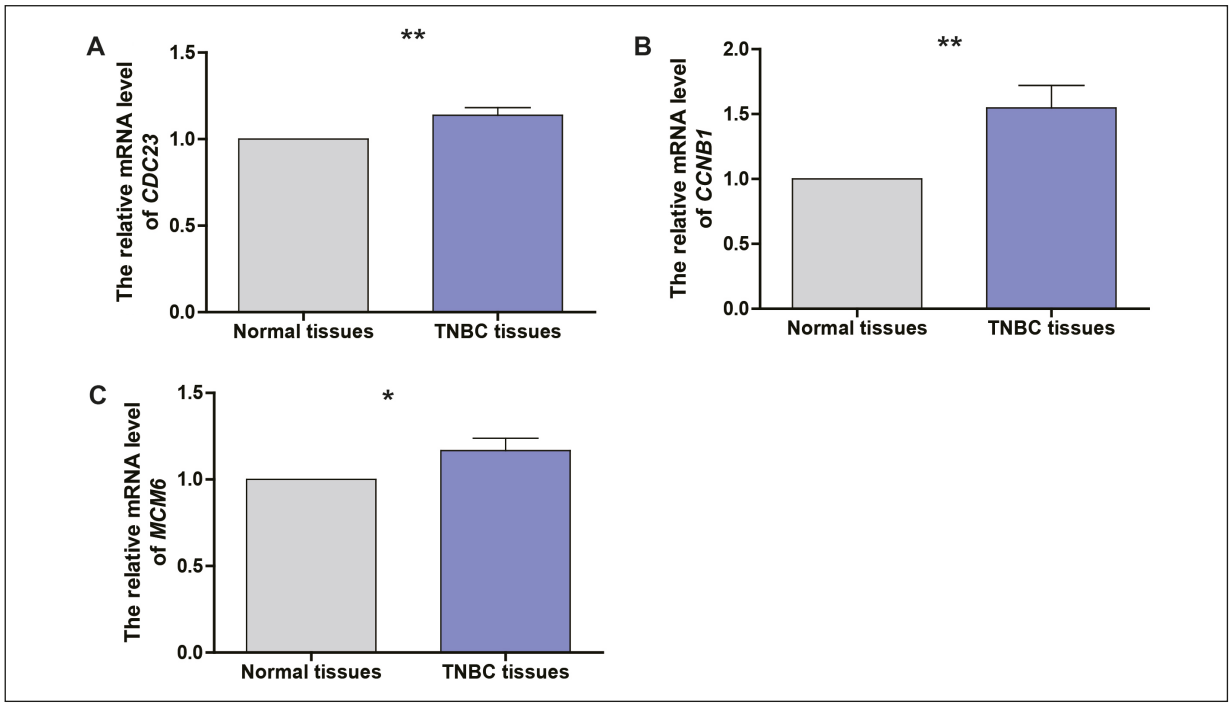

Figure 9. The expression of *MCM6*-related genes in 20 TNBC patients. **A-C**, The expression of (**A**) CDC23, (**B**) CCNB1, and (**C**) *MCM6* in 20 TNBC patients was detected by RT-PCR. *, $p < 0.05$; **, $p < 0.01$.

proliferation and invasion of lung cancer cells[59]. *MCM5* is highly expressed in renal cancer and is associated with malignant progression and poor prognosis[60].

The expression of *MCM6* is closely related to the occurrence and development of tumors and plays an important regulatory role in a variety of tumors[61-63]. In endometrial adenocarcinoma, *MCM6* expression correlates with histological grade and survival[64]. *MCM6* promotes hepatocellular carcinoma metastasis through the MEK/ERK pathway and can be used as a serum biomarker to identify the early recurrence of hepatocellular carcinoma[62]. Overexpression of *MCM6* is strongly associated with survival in glioma patients[65].

MCM7 is closely related to cell cycle regulation, transcription, and cell proliferation[66]. Existing studies[67] have shown that *MCM7* expression is elevated in various tumors, and overexpression of *MCM7* can promote tumor growth. In meningiomas, the level of *MCM7* protein expression in grade II was higher than that in grade I[68]. In papillary urothelial tumors, *MCM7* expression increased with tumor grade. *MCM7* is a risk factor for recurrence in Dukes stage C colorectal cancer patients[69]. *In vitro* studies[70] showed that low expression of *MCM7* significantly inhibited cell proliferation, colony formation, and migration in esophageal cancer cell lines. Abnormal proliferation has been identified as a crucial process in the evolution of TNBC. However, the roles of the *MCM2-7* in TNBC remain largely unknown.

Based on GEO datasets, we found that the expression of *MCM2-7* was increased in TNBC tissues compared to adjacent normal tissues. To further explore the prognostic significance of MCMs for TNBC patients, Kaplan-Meier analysis was applied to evaluate the correlation between the expression of *MCM2-7* and DFS. The Kaplan-Meier survival analysis revealed that TNBC patients with high expression of *MCM6* exhibited shorter survival time, while the expression of *MCM2, MCM3, MCM4, MCM5*, and *MCM7* were not related to TNBC patients' survival.

To explore the functions of *MCM6*, CancerSEA was performed for single-cell analysis. Functional correlation analysis of cancerSEA showed that the functional phenotypes of *MCM6* in breast cancer cells were positively correlated with cell cycle and DNA repair. Our GSEA data analysis also revealed that the cell cycle and DNA repair were significantly enriched in the *MCM6* high-expression group in TNBC. Our analysis of genetic screen data produced by the DepMap Project revealed a clear pro-proliferative role for

MCM6 in TNBC cells. Meanwhile, inhibition of proliferation was observed in MDA-MB-231 and BT-549 cells following *MCM6*-knockdown.

WGCNA analysis was performed to identify *MCM6*-highly correlated genes. Functional enrichment analysis showed that *MCM6*-related genes were enriched in the cell cycle, p53 signaling pathway, and DNA repair. As we know, MCM proteins are mainly known for their involvement in DNA replication and are expressed in rapidly dividing cells, but not in differentiated, aging, or quiescent cells. They are necessary for DNA replication initiation and progression in the cell cycle. Therefore, the upregulation of *MCM* gene members, DNA replication-promoting genes, results in dysregulated DNA replication in TNBC. To maintain the stability of genomics, cells have to synchronize DNA replication and damage repair with the cell cycle, and the p53 signaling pathway is central to this process. Previous studies showed that MCMs complex can directly interact with p53-binding protein-1 in HepG2 cells. Knockdown *MCM6* could significantly inhibit the foci formation of p53-binding protein-1 in HepG2 cell nuclei upon bleomycin-induced DNA damage[71]. These results indicated that the increased level of *MCM6*-related genes in TNBC is likely associated with the cell cycle, p53 signaling pathway, and DNA repair.

Finally, we detected the prognostic of the 41 *MCM6*-related genes and found that the increased levels of *CDC23*, *CCNB1*, *PTTG1*, *PCNA*, *CHEK1*, *CCNE2*, and *PLK1* were correlated with shorter DFS. Using LASSO Cox regression, 8 candidate survival-related genes were reduced to the 3 most powerful prognostic predictors, including *MCM6, CDC23*, and *CCNB1*, and a risk score model based on the *MCM6*-related genes was then generated. Meanwhile, a nomogram model that included risk score and other clinical factors was developed. The nomogram can predict the 1, 3, and 5-year survival rates precisely, thus providing evidence of treatment for TNBC patients.

Limitations

However, it is important to recognize several limitations of this study. Firstly, the analyses largely depend on publicly available gene expression databases, which might have heterogeneity in terms of patient selection, treatment history, and data collection methods. These variations can introduce biases that may impact the reproducibility and generalization of the findings. As the data utilized for this research are retrospective, causal inferences are limited. Prospective studies would be necessary to validate these findings and to establish a stronger cause-and-effect relationship between *MCM6* expression and TNBC pathogenesis/prognosis. Secondly, the *in vitro* effect of *MCM6* knockdown on cell proliferation, while significant, will require further validation using *in vivo* models to better understand the relevance of these findings. By acknowledging these limitations, the study presents an opportunity for future research efforts to build upon its findings, offering a more comprehensive understanding of the role of *MCM6* in TNBC.

Conclusions

In general, the study comprehensively analyzed the expression and prognosis of *MCM2-7* in TNBC. We found that *MCM6* was significantly overexpressed in TNBC and correlated with a worse prognosis. Knockdown of MCM6 significantly inhibited the proliferation of TNBC cells. Finally, the study has developed a risk score model composed of *MCM6*-related genes that can predict the prognosis of TNBC patients, which may help future treatment strategies.

Conflict of Interest

The authors declare that they have no competing interests.

Ethics Approval

The study was performed with the approval of the Ethics Committee of Fujian Provincial Hospital and complied with the Helsinki Declaration (No.: K2021-04-069).

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Authors' Contributions

Hui Zhang, Qinghui Wang, Yao Lin and Mengbo Lin contributed to the conception and design. Yidan Lin, Yue Yu and Xiaogeng Chen contributed to the development of methodology. Jinsi Wang, Huanhong Zeng and Qinghui Wang contributed to the writing, review, and/or revision of the manuscript.

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Data Availability
The corresponding author can provide the datasets used and/or analyzed during the current study upon reasonable request.

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