

Original Research

MCM6 is a Poor Prognostic Biomarker and Promotes Progression in Breast Cancer

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Abstract

Background: Breast cancer is the commonest global malignancy and the primary cause of carcinoma death. MCM6 is vital to carcinogenesis, but the pathogenesis of MCM6 remains unclear. **Methods:** MCM6 expression in patients with breast cancer was examined through The Cancer Genome Atlas (TCGA) database, immunohistochemistry, Quantitative Real-Time PCR (qRT-PCR) and Western blotting. The prognostic factors were assessed by the Kaplan–Meier method and Cox regression. On the basis of the key factors selected by multivariable Cox regression analysis, a nomogram risk prediction model was adopted for clinical risk assessment. The TCGA database was utilized to determine how MCM6 is correlated with chemotherapy sensitivity, immune checkpoint-related genes (ICGs), tumor-infiltrating immune cells, along with tumor mutation burden (TMB) and methylation. The impact of MCM6 on carcinoma cells was investigated in terms of proliferation, cell cycle as well as migrating and invasive behavior through CCK assays, flow cytometry, wound healing assays, Transwell assays and xenotransplantation experiments. **Results:** MCM6 expression was upregulated, which is closely associated with the size of the tumor ($p = 0.001$) and lymph node metastasis ($p = 0.012$) in patients with breast cancer. Multivariate analysis revealed MCM6 to be an independent risk factor for prognosis in patients with breast carcinoma. The nomograph prediction model included MCM6, age, ER, M and N stage, which displayed good discrimination with a C index of 0.817 and good calibration. Overexpression of MCM6 correlated with chemotherapy sensitivity, immune checkpoint-related genes (ICGs), tumor-infiltrating immune cells, tumor mutation burden (TMB), and methylation. Silencing MCM6 significantly inhibited proliferation, prolonged the G1 phase of the cell cycle, and restrained the proliferation, migration and invasive behavior of cancerous cells *in vitro* and inhibited tumor growth *in vivo*. **Conclusions:** Our research shows that MCM6 is highly expressed in breast cancer and can be used as an independent prognostic factor, which is expected to become a new target for the treatment of breast cancer in the future.

Keywords: breast cancer; MCM6; prognosis; nomogram; drug sensitivity; immune cell

1. Introduction

Breast cancer is the most common global malignancy and the primary cause of carcinoma death, severely damaging the health of the female population [1]. Breast cancer is a kind of disease with high heterogeneity in morphology, molecular biology, clinical manifestations and therapeutic response that leads to prevention and treatment remaining challenging worldwide [2–4]. In recent years, many genes related to the progression of breast cancer have been discovered according to its pathogenesis and drug resistance mechanism. Traditional chemotherapy combined with targeted therapy and immunotherapy achieved better effects than before. For instance, trastuzumab, a monoclonal antibody utilized as a standard treatment for malignancy in the case of Human epidermal growth factor receptor-2 (HER2) overexpression in cancer cells, has an objective response rate of 26% in clinical treatment [5]. The combined medication of cetuximab and cisplatin could inhibit Epidermal Growth Factor Receptor (EGFR) and prolong progression-

free survival by 1.5 to 3.7 months and overall survival (OS) time extended from 9.4 to 12.9 months [6]. Targeted therapy is of vital importance for the overall prevention and treatment of malignancy. However, continued treatment results in a loss of target expression, and gene mutations downstream of the target gene itself lead to acquired resistance [7–9], which are the greatest challenge facing targeted therapies. Immune checkpoint inhibitors (ICIs) have entered the treatment regimen for metastatic breast cancer. Biomarkers such as PD-L1 expression and tumor infiltrating lymphocytes (TIL) are commonly used for immunotherapy, yet only a subset of patients benefit from ICIs and immune-based combination therapy [10]. Immunotherapy alone has been shown to increase the risk for early death compared with other treatments. Immunotherapy combined strategy can prevent early death [11]. Based on the combination of ICIs with each other or with other anticancer drugs, including systemic chemotherapy and poly ADP-ribose polymerase (PARP) inhibitors, targeted therapy has



become a widely used strategy and new direction in cancer immunotherapy [12]. Identification of biomarkers predicting response to immune combinations is the key to progress [13]. Therefore, the discovery of novel diagnostic and therapeutic biomarkers could be a breakthrough in the treatment of the disease.

MCM6 is a key regulator of DNA replication and hence plays an undeniable role in maintaining the cell cycle [14]. MCM6 expression is upregulated in many cancerous cells, and the overexpression of MCM6 promotes the occurrence and development of carcinoma [15–18]. Relevant studies proved that MCM6 regulates proliferation, immune response and maintenance of the DNA replication system and tumor cell proliferation and invasion and other physiological and pathological processes [19,20]. MCM6 promotes metastasis through the MEK/ERK pathway and serves as a novel serum biomarker for early relapse in hepatocellular carcinoma [21]. MCM6 is highly expressed in tumors with higher histological grades, especially in clinically aggressive breast cancers (e.g., luminal B, HER2-positive, and triple-negative subtypes) [16]. Therefore, MCM6 might represent a sensitive and specific biomarker for cancer prognosis. Inhibition of MCM6 is likely to be an effective treatment. Nevertheless, the function and related mechanism are unclear in breast carcinoma.

This study aimed to explore the correlation between the expression level of MCM6 and clinicopathological features and to evaluate whether MCM6 can be used as a prognostic factor for breast cancer. Then, the related signaling pathways and chemotherapy sensitivity with MCM6 overexpression in patients with breast cancer were evaluated via gene set enrichment analysis (GSEA) and Genomics of Drug Sensitivity in Cancer (GDSC) database. Additionally, The Cancer Genome Atlas (TCGA) database was employed to determine the correlation between MCM6 and tumor-infiltrating immunocytes. The functional role of MCM6 was further investigated *in vitro* and *in vivo* in breast cancer cells. This study provides an important basis for further exploring the role of MCM6 in diseased cells and for targeted treatment of breast carcinoma.

2. Materials and Methods

2.1 Cell Culture and Clinical Specimens

The human breast cancer cell lines MCF-7, BT549, T47-D, MDA-MB-231, and HCC-1806 and the human breast epithelial cell line MCF-10A were obtained from The Cell Bank of Chinese Academy of Sciences and ATCC and American Type Culture Collection (ATCC), and cultured at 37 °C with 5% CO₂. MCF-7 and T47D are estrogen receptor positive human breast cancer cell lines, BT549, MDA-MB-231 and HCC-1806 human triple negative breast cancer cell lines. All experiments were performed using mycoplasma-free cells. All cell lines were authenticated using single nucleotide polymorphisms profiling. One hundred cases of paraffin-embedded diseased tissues and corre-

sponding paired normal tissues were obtained from hospitalized patients with breast carcinoma at the First Affiliated Hospital of Kunming Medical University from September 2014 to July 2021. All paraffin tissue samples were kept by our hospital and were used for immunohistochemical staining, and there was no need to consult the patient. This study was approved by the relevant authoritative organization of the hospital.

2.2 Quantitative Real-Time PCR (qRT-PCR) and Western Blotting

TRIzol reagent (15596-026, Invitrogen, Carlsbad, CA, USA) was utilized for the extraction of the aggregate RNA, which was reverse transcribed to cDNA according to the requirements of the RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific, Waltham, MA, USA). PCR amplification was conducted using the SYBR Green Select Master Mix System (4472908, Invitrogen, Carlsbad, CA, USA) on a CFX96TM Real-Time PCR Detection System. The target mRNA expression was normalized to the mRNA expression of GAPDH. The primers were as such follows: MCM6: forward, 5'-TGTCAGTGGTGTGATGGATATG-3', reverse 5'-GCTGTCTGTTCTCATCTCTG-3'; GAPDH: forward, 5'-GGCAACAATATCAGCAACTTC-3', reverse 5'-GGTCCTTAGGTGTGGTATC-3'. All qRT-PCR experiments were conducted no less than three times. MCM6 expression was examined via Western blotting. Briefly, the cells were lysed for 20 min using RIPA buffer (89900, Thermo Fisher Scientific, Waltham, MA, USA), and all cell proteins were extracted after centrifugation. The BCA Protein Assay Kit (23227, Pierce, Waltham, MA, USA) was adopted for the quantification of cell lysate protein. The protein solution was separated using SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane, which was treated with evaporated milk (5%) at 4 °C for a night. The PVDF membrane was cultured with anti-MCM6 antibody (13347-2-AP, Proteintech, Rosemont, IL, USA) at 1:1000 at approximately 23 °C and treated with secondary antibody at a 1:10,000 dilution. The internal control used was β -actin (ZSGB-BIO, Beijing, China, #TA-09).

2.3 Immunohistochemical (IHC) Staining

IHC staining was performed in conformity with the manufacturer's protocol (Dako REAL EnVision Detection System; Dako, Santa Clara, CA, USA). To elaborate, the tissue segments underwent a baking process at 68 °C for 2 h. The slices were dewaxed with xylene, gradient alcohol and distilled water. Then, the slices were boiled in citric acid buffer (pH 6.0, 0.01 M) for 2 min at high temperature and pressure for antigen repair. The slices were incubated with 0.3% hydrogen peroxide to block endogenous peroxidase activity. Subsequently, the slices were cultured with MCM6 antibody (diluted 1:400; 13347-2-AP, Proteintech) at 4 °C overnight and goat anti-mouse IgG-HRP antibody.

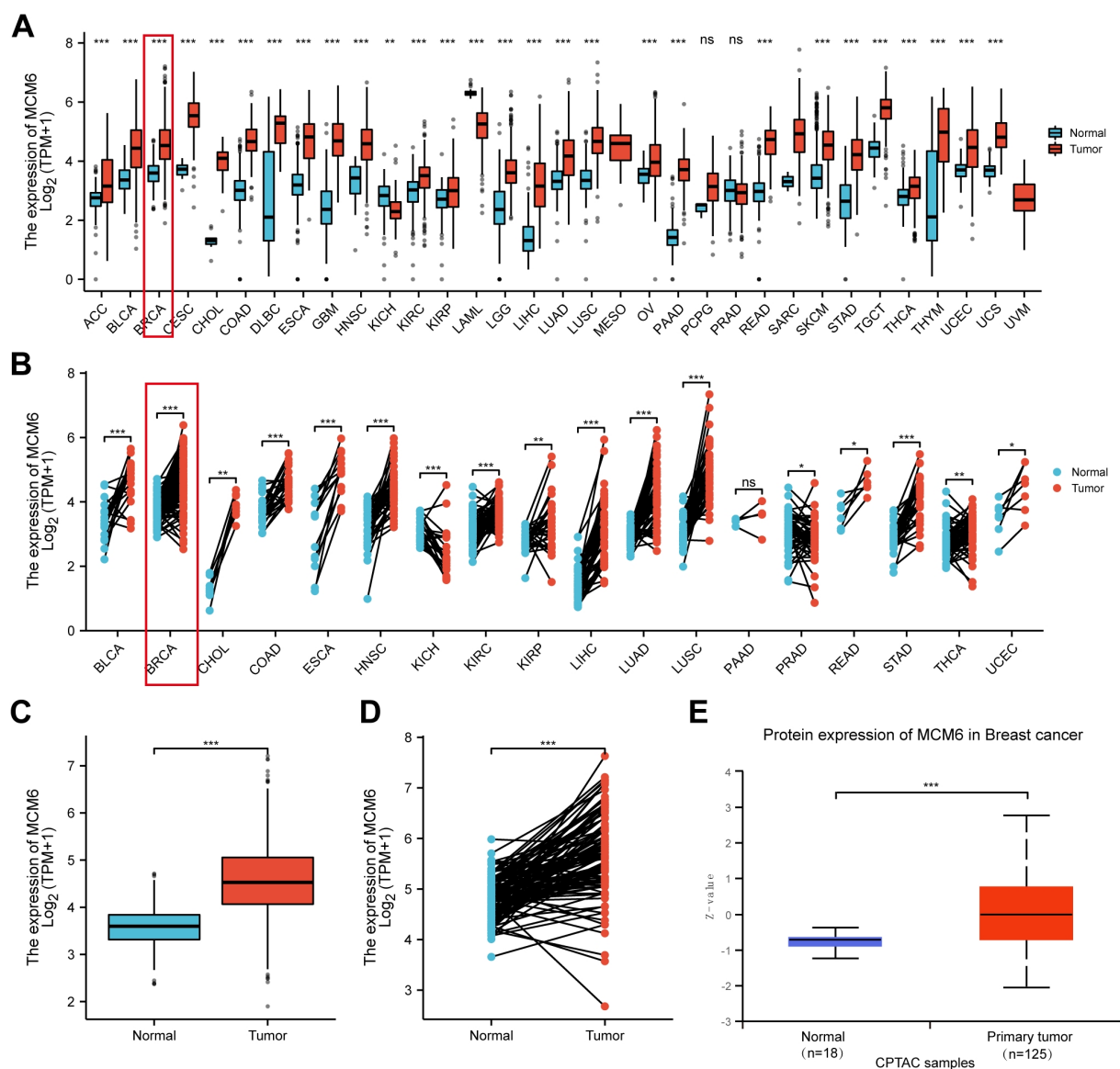


Fig. 1. MCM6 was significantly upregulated in breast cancer based on database analysis. (A,B) MCM6 expression was increased in a variety of tumors according to The Cancer Genome Atlas (TCGA) database analysis. (C) Box plot of TCGA database describes the different expression levels of MCM6 between normal tissues and cancerous tissues. (D) Based on the same number of samples, MCM6 expression in normal and diseased tissues was compared. (E) MCM6 protein expression was higher in normal breast tissue according to the UALCAN database. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. ns, not significant; CPTAC, Clinical Proteomic Tumor Analysis Consortium.

Diaminobenzidine tetrahydrochloride (DAB) was used to observe the reaction. IHC staining was scored by two independent investigators. The aggregate score was obtained by multiplying the proportion of positive tumor cells [scored as 0 (<5%), 1 (6~25%), 2 (26~50%), 3 (51~75%), 4 (76~100%), respectively] and the staining intensity (scored as 0, 1, 2, and 3 according to severity). Based on the final scores, less than 5% of cells were stained, regardless of intensity, and specimens were categorized as negative; weak, moderate and strong were given marks of 1~4 points, 5~8 points and 9~12 points, respectively. For the following statistical analysis, the negative and weak groups were taken

as the low MCM6 expression group, and the moderate and strong groups were considered to be the high MCM6 expression group.

2.4 Data from the TCGA Cohort and UALCAN Platform

The RNA-seq data and relevant information about the research subjects were acquired from the 2022 version of The Cancer Genome Atlas database (TCGA) (<https://www.genome.gov/Funded-Programs-Projects/Cancer-Genome-Atlas>) from the official website. The subjects were grouped into two groups according to expression level, namely, the median MCM6 mRNA

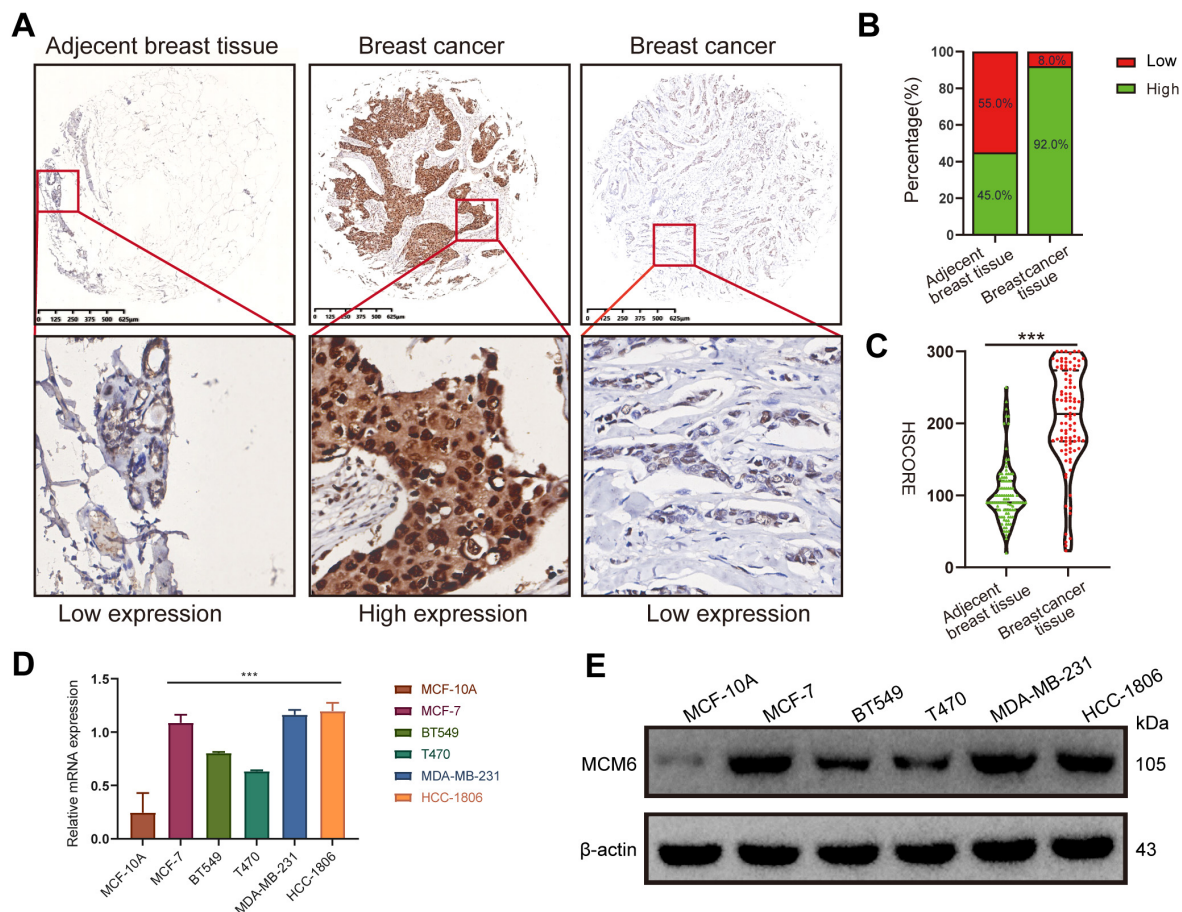


Fig. 2. MCM6 is significantly up-regulated in tissues and cells of breast cancer based on experimental verification. (A–C) Immunohistochemical staining of MCM6 expression in adjacent breast tissue and breast cancer tissues, representative immunohistochemical images and statistical results (n = 100). (D,E) Quantitative Real-Time PCR (qRT-PCR) and western blotting analyses of MCM6 expression in MCF-10A breast cells and diseased cells, including MCF-7, BT549, T470, MDA-MB-231, and HCC1806 breast cancer cells. Statistics are presented as the means ± SDs. *** $p < 0.001$.

expression of each sample. The clinicopathological data of the subjects were collected, including age, clinical classification of primary focus, regional lymph node and distant metastasis, represented by TNM collectively, endogenous molecular classification (Luminal A, Luminal B, basal like, HER2 overexpression or normal type) and histological type. The expression level of MCM6 protein in breast cancer patients was obtained in UALCAN database (<http://ualcan.path.uab.edu/>).

2.5 Prognostic Analysis and Construction of a Predictive Nomogram

The online websites Kaplan–Meier Plotter (<http://kmplot.com/analysis/>) and PrognScan (<http://dna00.bio.kyutech.ac.jp/PrognScan/>) were selected for the prognostic analysis of MCM6 in patients with breast carcinoma. The “rms” R package was used to combine MCM6 expression with clinicopathological features to construct a nomogram risk prediction model for the prediction of OS, and the predictive performance was measured using calibration curves and the C-index [22,23].

2.6 Gene Set Enrichment Analysis (GSEA)

The h.all.v7.5.symbols.gmt [Hallmarks] gene set database was selected. GSEA was deployed to elaborate the functional differences of the gene between the two expression groups, and the MCM6 expression level was considered a label of phenotype. The arrangements of gene sets were arranged out by 1000 times per every analysis. The False Discovery Rate (FDR) q value, the normalized enrichment score (NES) and the nominal p value were established to classify each phenotypic enrichment signaling pathway.

2.7 Correlation Analysis between MCM6 Expression and Immune Checkpoint-Related Genes in Breast Cancer

With the RNA-seq data, the MCM6-ICG relations to explore the immune genes related to MCM6 expression were analyzed using the R package, which includes limma, reshape2, ggplot2, ggpubr, and corplot. The correlation threshold was set to a correlation coefficient ≥ 0.2 and a p value less than 0.05.

2.8 Cell Proliferation Assay

Diseased cell proliferation was measured with a sulforhodamine B (SRB) assay. The cells were seeded in a 96-well plate (100 μ L/well) and fixed with cold 10% trichloroacetic acid for half an hour at room temperature. The SRB solution (0.4%, Sigma S9012, Wappingers Falls, NY, USA) was treated with deionized water and then added for dyeing for approximately 10 min. Tris solution (10 mM) was incubated at 23 °C for 5 min, and the optical density (OD) of the cells was determined at 490–530 nm wavelengths [24].

2.9 Cell Cycle Assay

The cell samples were collected using trypsin, fixed with 80% ethanol at 4 °C in a dark environment overnight, and then stained in the dark with propidium iodide (0.1 mg/mL, BD Biosciences, Franklin Lake, NJ, USA) and RNase A (1 mg/mL) for 30 minutes. The cell cycle of each group was tested via flow cytometry. The data were analyzed by FlowJo 7.6 (San Carlos, CA, USA) [25].

2.10 Cell Apoptosis Assay

The collected cells were resuspended in binding buffer, cultured with Annexin V-FITC (BD Biosciences) for 10 minutes in a dark environment at room temperature and subsequently stained with binding buffer and PI (BD Biosciences) in darkness for 30 minutes at 4 °C. Cell measurement was conducted using flow cytometry [25].

2.11 Wound Healing Assay

The cells were inoculated into 6-well culture plates in complete medium until they were 80% confluence. The cells were scratched with a 200 μ L pipette tip. Then the medium was changed to a serum-free medium. The migration process was carefully observed at 0, 24 and 48 h on an inverted microscope (Olympus, Tokyo, Japan). ImageJ (National institutes of Health, Bethesda, MD, USA) was employed to calculate the migration area [25].

2.12 Transwell Assay

The cell suspension was cultured in serum-free medium into the upper layer of the experimental plates coated with Matrigel (BD Biosciences). The serum-containing medium was placed in the lower layer. The cells that invaded the lower layer received methanol treatment and Giemsa staining and were photographed microscopically [26].

2.13 Animal Experimental Procedures

The animal experiments won approval from the authoritative organization of the hospital. Six-week-old female BALB/C nude mice were maintained in a pathogen-free environment. MDA-MB-231 cells (1×10^6 /point), which were divided into two groups (Normal control group, shMCM6 group), were injected subcutaneously into the

Table 1. Correlation between MCM6 expression and clinicopathologic parameters in breast cancer.

Characteristics	MCM6 expression		<i>p</i> value
	Low (%)	High (%)	
Age			0.309
<60	7 (63.6)	69 (77.5)	
≥ 60	4 (36.4)	20 (22.4)	
History grade			0.427
1	1 (9.1)	2 (2.2)	
2	8 (72.7)	65 (73.0)	
3	2 (18.2)	22 (24.7)	
Tumor diameter			0.001
≤ 20 mm	7 (63.6)	14 (15.7)	
>20 mm, ≤ 50 mm	3 (27.3)	58 (65.2)	
>50 mm	1 (9.1)	17 (19.1)	
Lymph node metastasis			0.012
With	8 (72.7)	30 (33.7)	
Without	3 (27.3)	59 (66.3)	
Vascular invasion			0.547
With	6 (54.5)	40 (44.9)	
Without	5 (45.5)	49 (55.1)	
Distant Metastasis			0.074
With	10 (90.9)	57 (64.0)	
Without	1 (9.1)	32 (36.0)	
Surrogate subtypes			0.836
Luminal A-like	2 (18.2)	9 (10.1)	
Luminal B-like	4 (36.4)	42 (47.2)	
Her-2+	3 (27.3)	22 (24.7)	
Triple negative breast cancer	2 (18.2)	16 (18.0)	

mammary fat pads of the mice. The subcutaneous tumor size was observed every 4 days. The tumor size was measured using a caliper, and the formula was as follows: volume = $1/2$ (width² $\times$ length). The mice were sacrificed after 24 days. The diseased tissues were excised and weighed. The tumors were collected and used for additional IHC pathological analysis [25].

2.14 Statistical Analysis

For data analysis, SPSS 22.0 software (IBM Corp., Armonk, NY, USA) was adopted. The expression difference between breast tumor and normal breast tissues was analyzed by the Wilcoxon signed-rank test. Univariate and multivariate Cox analyses were selected to analyze the influence of MCM6 expression along with other factors on mouse survival. Logistic regression analysis was performed to determine how MCM6 was related to other clinical factors. One-way analysis of variance was employed to make comparisons between two groups. $p < 0.05$ was deemed to be statistically significant.

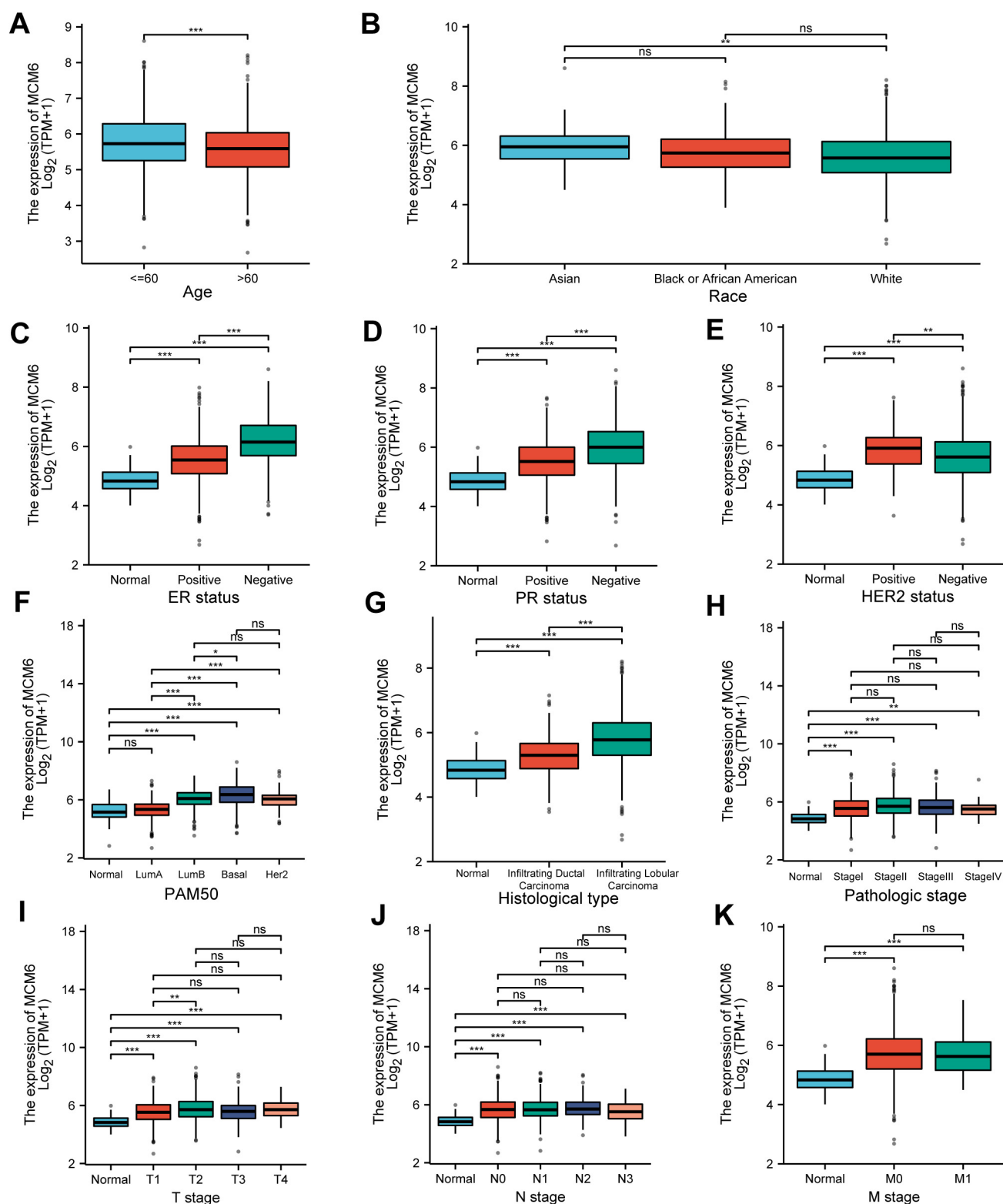


Fig. 3. Expression of MCM6 in different clinicopathological features. (A) MCM6 expression in people under 60 years old and over 60 years old. (B) MCM6 expression in Asian, Black or African American and White individuals. (C–E) Relationship between MCM6 expression and Estrogen Receptors (ER), Progesterone Receptors (PR), and Human Epidermal Growth Factor Receptors 2 (HER2) status. (F) Relationship between MCM6 expression and different cancerous types of molecules. (G) Relationship between MCM6 expression and different histological subtypes of breast cancer. (H) MCM6 expression in different pathologic stages. (I–K) MCM6 expression in the T, N and M phases. Statistics are presented as the means $\pm$ SDs. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns, not significant.

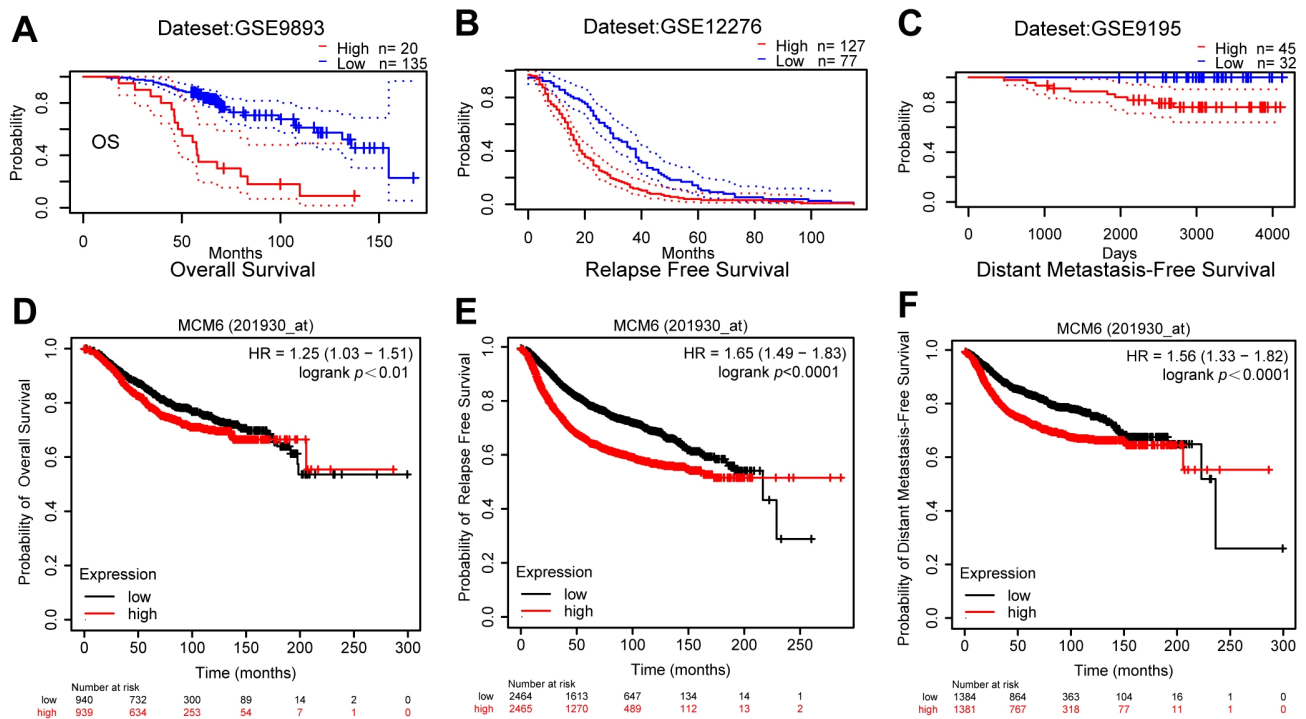


Fig. 4. Breast cancer with high MCM6 expression has a poor prognosis. The Prognoscan online website was selected to analyze the prognostic role of MCM6 in breast carcinoma, and (A), (B), and (C) represent Overall Survival (OS), Relapse Free Survival (RFS) and Distant Metastasis Free Survival (DMFS), respectively. The Kaplan Meier Plotter online website was also utilized for the analysis of the prognostic effects of MCM6 in breast cancer, and the results suggested reduced survival time in patients with overexpression of MCM6 in terms of all three indicators mentioned above, and (D), (E), and (F) represent OS, RFS and DMFS, respectively.

3. Results

3.1 MCM6 Expression was Significantly Increased in Breast Cancer

We first explored MCM6 expression in a wide range of human malignancies. The mRNA expression of MCM6 increased in various cancers from TCGA database analysis (Fig. 1A,B), and MCM6 expression in breast cancer considerably exceeded that in normal tissues (Fig. 1C,D). The protein expression level of MCM6 in patients with breast cancer outstripped that in normal tissues from the online database UALCAN (<http://ualcan.path.uab.edu/>) (Fig. 1E). Consistent with the expression pattern from the online database, immunohistochemical staining outcomes indicated higher MCM6 expression in diseased tissues than in adjacent breast tissue. Among 100 cases of breast cancer tissues, 92 cases (92.0%) had overexpression of MCM6, and the rest (8.0%) had low expression of MCM6, while there were 45 cases (45.0%) with overexpression of MCM6 and 55 cases (55.0%) with low expression of MCM6 in adjacent tissues (Fig. 2A–C). In comparison with MCF-10A human breast epithelial cells, MCM6 expression in breast cancer cell lines (MCF-7, BT549, T470, MDA-MB-231, HCC1806) was significantly increased (Fig. 2D,E). These results suggest that MCM6 was overexpressed in patients with breast carcinoma.

3.2 MCM6 is a Poor Prognostic Factor in Breast Cancer

We further explored how MCM6 expression is correlated with clinicopathologic parameters (age, histological grade, size of tumor, lymph node metastasis, vascular invasion, distant metastasis, and molecular typing). The outcomes demonstrated that MCM6 expression was associated with the size of tumor ($p = 0.001$) and lymph node metastases ($p = 0.012$) (Table 1). An analysis of the TCGA database revealed that the expression of MCM6 varied according to age, and there were differences between Asians and Caucasians White (Fig. 3A,B); MCM6 expression in estrogen receptors (ER) and progesterone receptors (PR)-negative breast cancer patients was higher than that in ER- and PR-positive breast cancer patients (Fig. 3C,D); MCM6 expression in HER2-positive subjects was more obvious than in negative breast cancer patients (Fig. 3E); MCM6 expression was elevated in luminal B, basal and HER2 breast cancer (Fig. 3F); MCM6 expression in invasive lobular carcinoma was higher than that in invasive lobular carcinoma tube carcinoma (Fig. 3G); no notable difference in the expression of MCM6 was found in each clinical stage (Fig. 3H); MCM6 expression was different only in the T1 and T2 stages; no considerable difference was shown in MCM6 expression between the N stage and the M stage, which T represents tumor size stage, N represents lymph node metastasis stage, and M represents distant

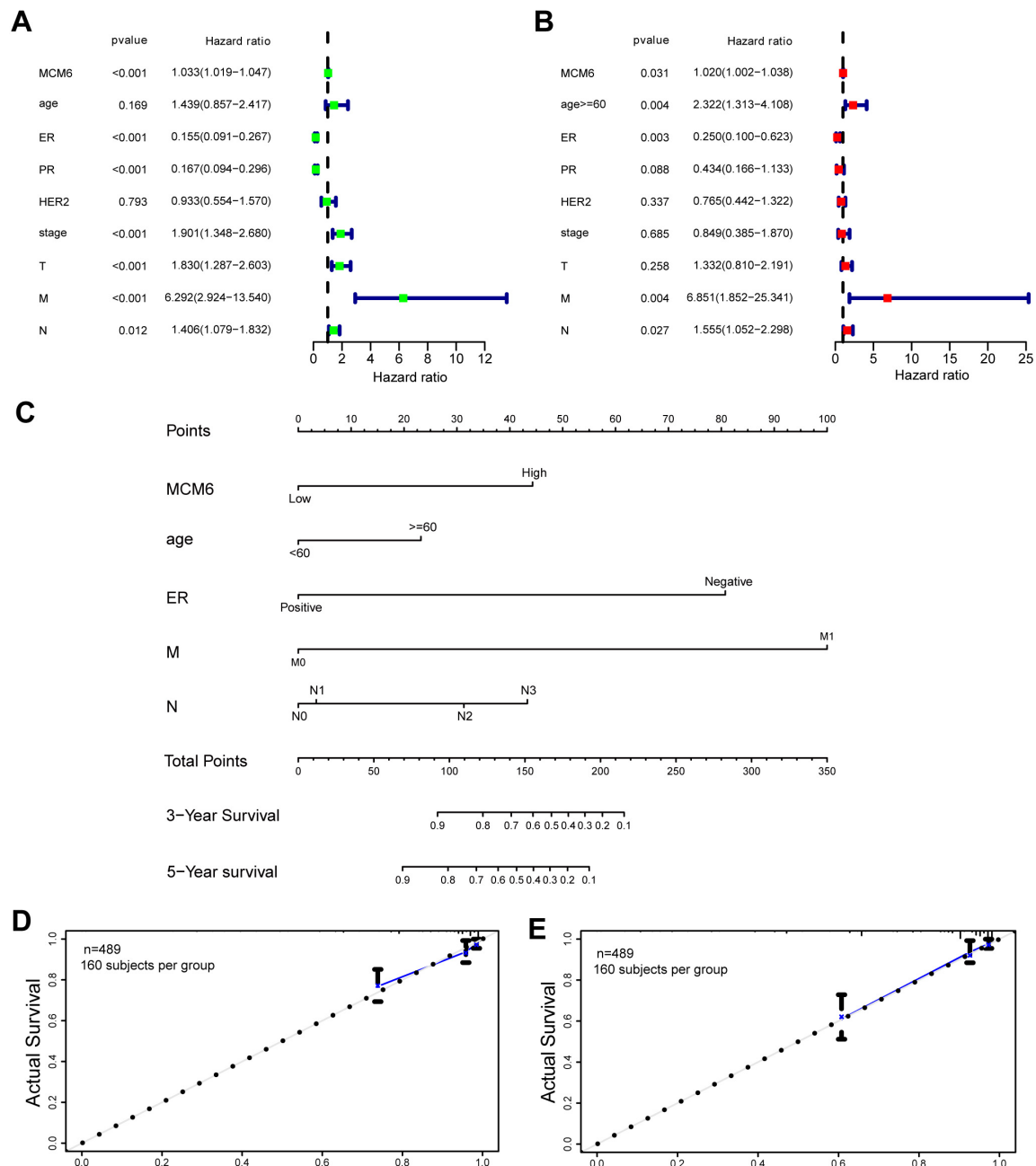


Fig. 5. Univariate and multivariable Cox regression analysis and construction of nomograms related to patient prognosis. (A) The prognosis of MCM6 was analyzed by univariate Cox regression. (B) Multivariate Cox regression analysis of the prognosis of MCM6. (C) The nomogram combined with the risk signature and clinicopathological factors. (D,E) Calibration plots for the prediction of prolonged survival of three and five years.

metastasis stage (Fig. 3I–K). Based on the analysis from the Prognoscan online website, breast cancer patients with high MCM6 expression had reduced OS, relapse-free survival (RFS) and distant metastasis-free survival (DMFS) (Fig. 4A–C), which is consistent with the results of the Kaplan-Meier Plot online prognostic analysis (Fig. 4D–F).

In addition, univariate Cox regression analysis revealed that the expression of ER, PR, stage, T stage, M stage, N stage and MCM6 was associated with a low survival rate of patients (Fig. 5A). In multivariate Cox regres-

sion, after adjusting for other confounding factors, the expression of age, ER, M, N and MCM6 was an independent indicator (Fig. 5B). Furthermore, various indicators (age, ER, M, N and MCM6 expression) were included in the nomogram prediction model by multivariate Cox regression. The total score was obtained by age, expression of ER, M stage, N stage, MCM6, and prediction of survival probabilities at three years and five years (Fig. 5C). The C-index for the prediction nomogram was 0.817. Calibration curves displayed the predictive power of the predic-

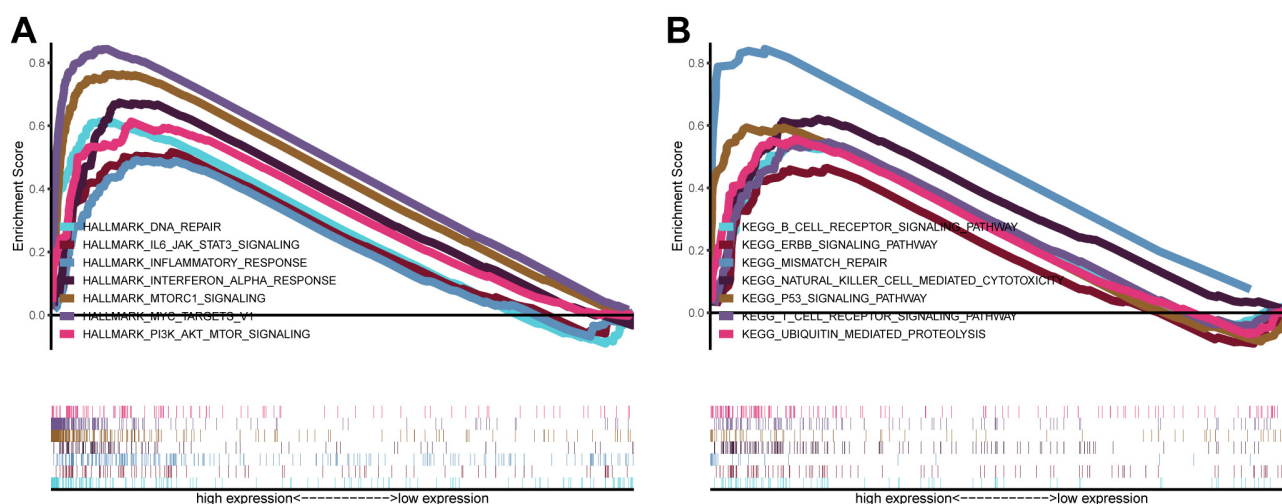


Fig. 6. Analysis of signaling pathways by using GSEA. (A) GSEA plots showing the NES for HALLMARK, which had close relations to the overexpression of MTM6 in breast carcinoma and involved signaling pathways, including DNA repair, signaling types of IL6 JAK STAT3, MTORC1 and PI3K AKT MTOR, inflammatory and interferon alpha responses, and MYC targets V1. (B) NES for KEGG, which has a close association with the overexpression of MTM6, including B-cell receptor, ERBB, p53, T-cell receptor signaling pathways, mismatch repair, natural killer cell-mediated cytotoxicity, and ubiquitin-mediated proteolysis.

tive model in terms of survival time in patients with breast carcinoma (Fig. 5D,E). These results suggest that overexpression of MCM6 is a dismal prognostic factor for breast cancer.

3.3 Enrichment and Functional Analysis of Gene Sets

GSEA was performed between subgroups with different levels of MCM6 expression using the whole gene network based on the TCGA dataset with a view to explore the biological pathways via which MCM6 works in diseased tissues. The biological processes involved mainly included DNA repair, different pathways of IL6-JAK-STAT3, MTORC1, PI3K-AKT-MTOR, MYC, etc. (Fig. 6A). KEGG analysis demonstrated that overexpression of MCM6 was mainly involved in the process of mismatch repair, the regulation of ubiquitination proteolysis, ERBB signaling pathway and p53 signaling pathway. In addition, MCM6 is enriched in immune signaling pathways and activates related immune responses, such as B cell and T cell receptor pathways, and cytotoxic effects of natural killer cells (Fig. 6B).

3.4 Evaluated MCM6 Expression and Sensitivity to Chemotherapy Drugs

The impact of MCM6 expression on chemosensitivity in patients with breast carcinoma were assessed via the pRRophetic algorithm and the half-maximal inhibitory concentration available in the Genomics of Drug Sensitivity in Cancer (GDSC) database. The results demonstrated that 134 chemotherapeutic drugs were identified to be significantly correlated with MCM6 expression. In the group overexpressing MCM6, we found that there were 14 chemotherapy drugs with significant effects (Fig. 7A–N),

especially PD-0332991 ($p = 3.7 \times 10^{-12}$) and trametinib ($p < 2.22 \times 10^{-16}$). According to these findings, the drug sensitivity of these small molecule compounds is better when MCM6 is highly expressed, but further exploration and experimental verification are needed.

3.5 Correlation Analysis of MCM6 and ICGs, Immunity, TMB and Methylation

The expression correlation between MCM6 and genes related to immune detection points was analyzed based on the TCGA BRCA database. Our research outcomes indicated a positive correlation between MCM6 and the expression of multiple immune checkpoints and a negative correlation with that of BTNL9 (Fig. 8A). Analysis of the relationship between MCM6 and immunity, including 24 kinds of immune cells, showed a significantly negative correlation with eight immune cells (iDC, CD8 T cell, Th17 cells, eosinophils, mast cells, NK CD56 bright cells, NK cells, pDC) but a significantly positive correlation with fifteen immune cells (cells of Th2, aDC, Th1, Treg, T helper, NK CD56dim, Macrophages, B, Tcm, DC, Tgd, T, Term, Neutrophils, TFH) (Fig. 8B). In addition, MCM6 expression was positively correlated with TMB ($R = 0.33$, $p < 2.2 \times 10^{-16}$) (Fig. 8C). Finally, we also analyzed the methylation levels of MCM6 in the diseased group and the normal group, and the outcomes revealed that MCM6 had a higher methylation level in breast cancer (Fig. 8D).

3.6 MCM6 Knockdown Inhibited the Proliferation of Breast Cancer Cells

To make a clarification of the biological role of MCM6 in the progression and prognosis of breast carcinoma, the lentivirus system was used to conduct stable MCM6

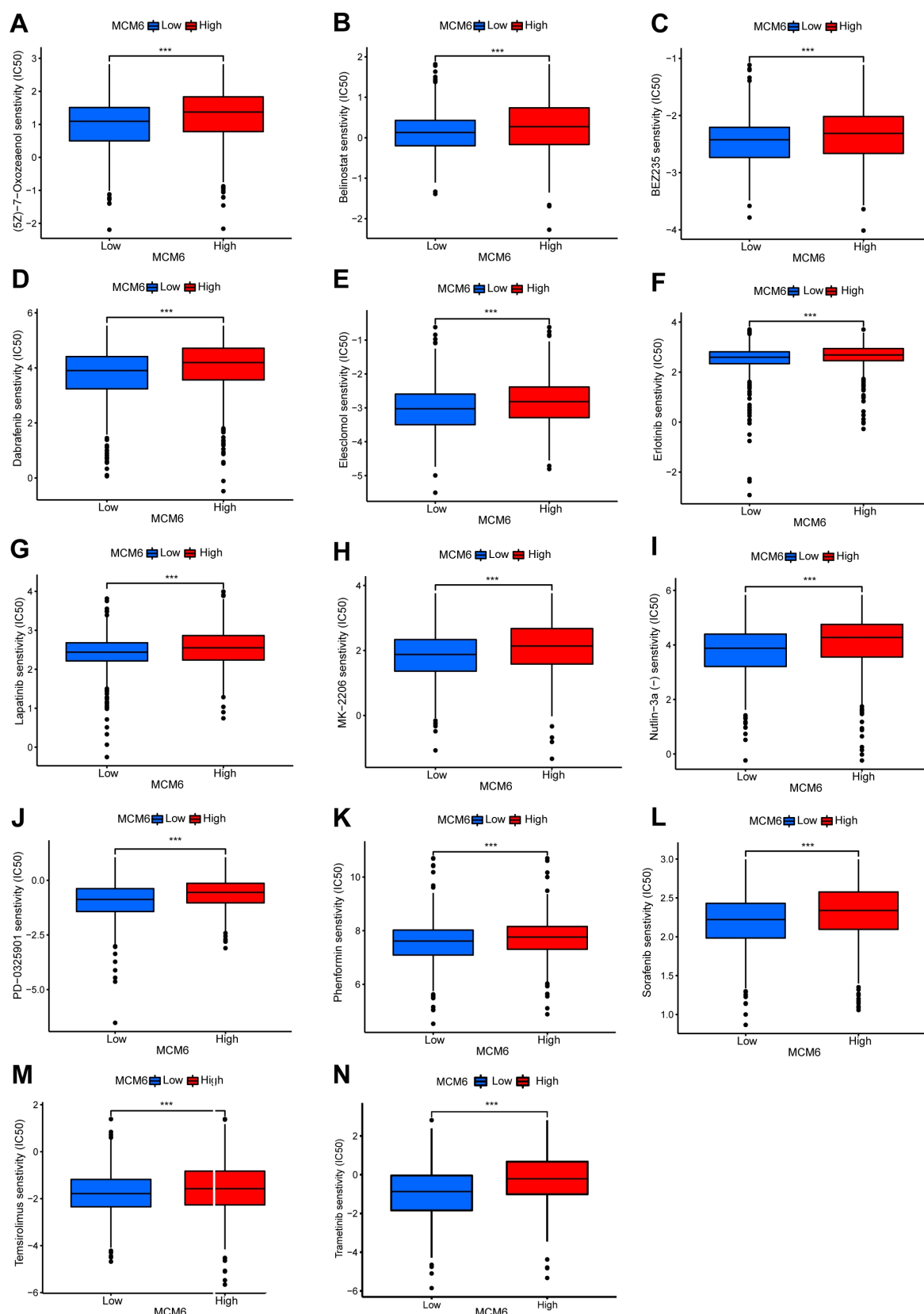


Fig. 7. MCM6 expression correlates with the chemosensitivity of breast cancer. Box plots show the chemosensitivity of (5Z)-7-oxozeaenol (A), belinostat (B), BEZ235 (C), dabrafenib (D), elesclomol (E), erlotinib (F), lapatinib (G), MK-2206 (H), Nutlin-3a (I), PD-0325901 (J), phenformin (K), sorafenib (L), temsirolimus (M) and trametinib (N) in high- and low-expression of MCM6 in breast cancer. *** $p < 0.001$.

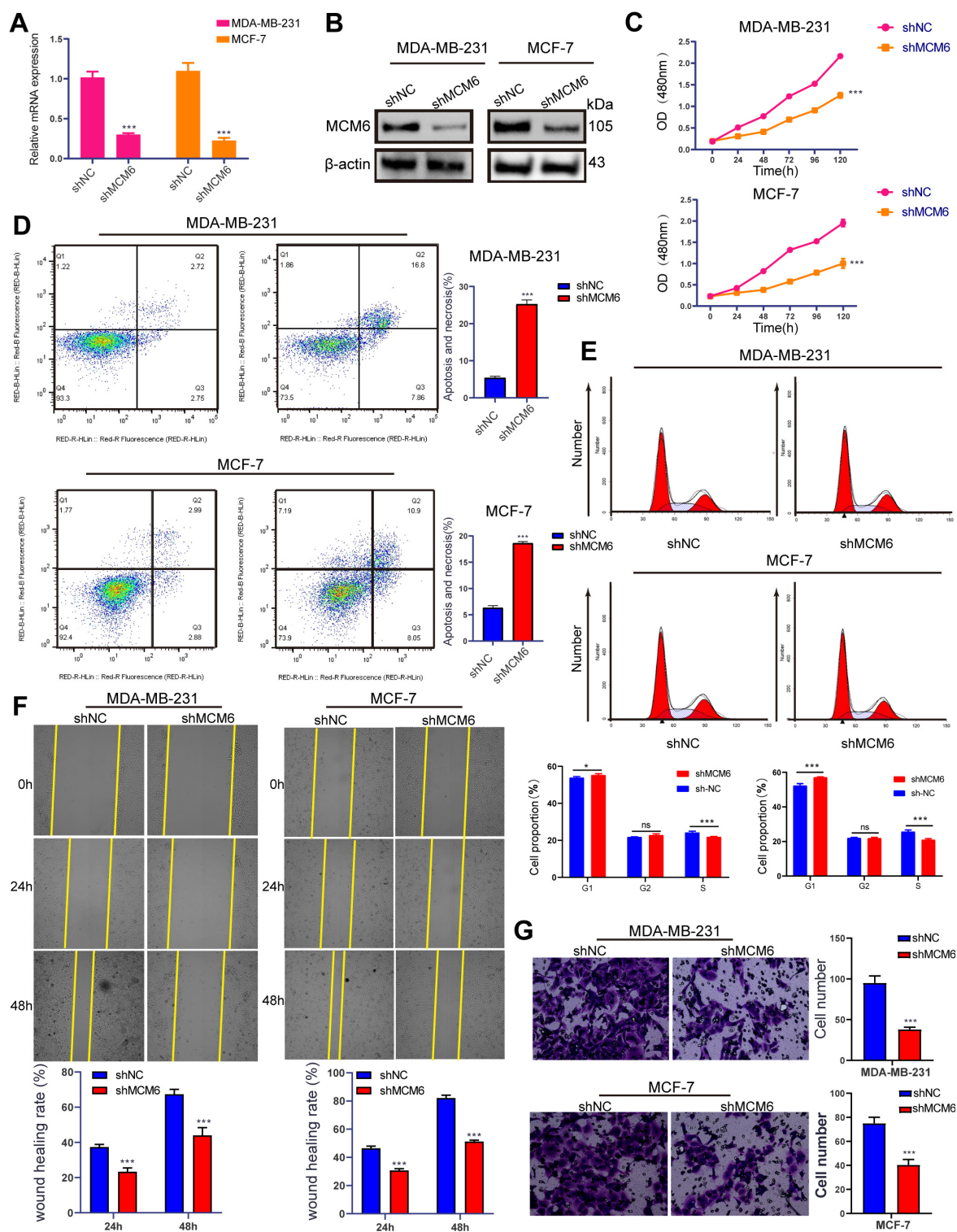


Fig. 9. MCM6 promotes breast cancer cell growth, migration, and invasion and inhibits apoptosis *in vitro*. (A,B) qRT-PCR and WB showing MCM6 expression in stably silenced in MDA-MB-231 and MCF-7 cells. (C) CCK8 experiment results show that inhibition of MCM6 significantly inhibits the proliferation of MDA-MB-231 and MCF-7 cells. (D) Inhibition of MCM6 significantly promotes the apoptosis rate of MDA-MB-231 and MCF-7 cells. (E) Knockdown MCM6 leads to a large number of G1 stage cancer cells to multiply, and S stage cancer cells to reduce. (F) Wounding healing assay showed that knockdown of MCM6 significantly slowed down the wound healing rate of MDA-MB-231 and MCF-7 cells. (G) Invasion chamber experiment confirmed that knockdown of MCM6 significantly inhibited the invasion rate of MDA-MB-231 and MCF-7 cells. Statistics are presented as the means $\pm$ SDs. * $p < 0.05$, *** $p < 0.001$; ns, not significant.

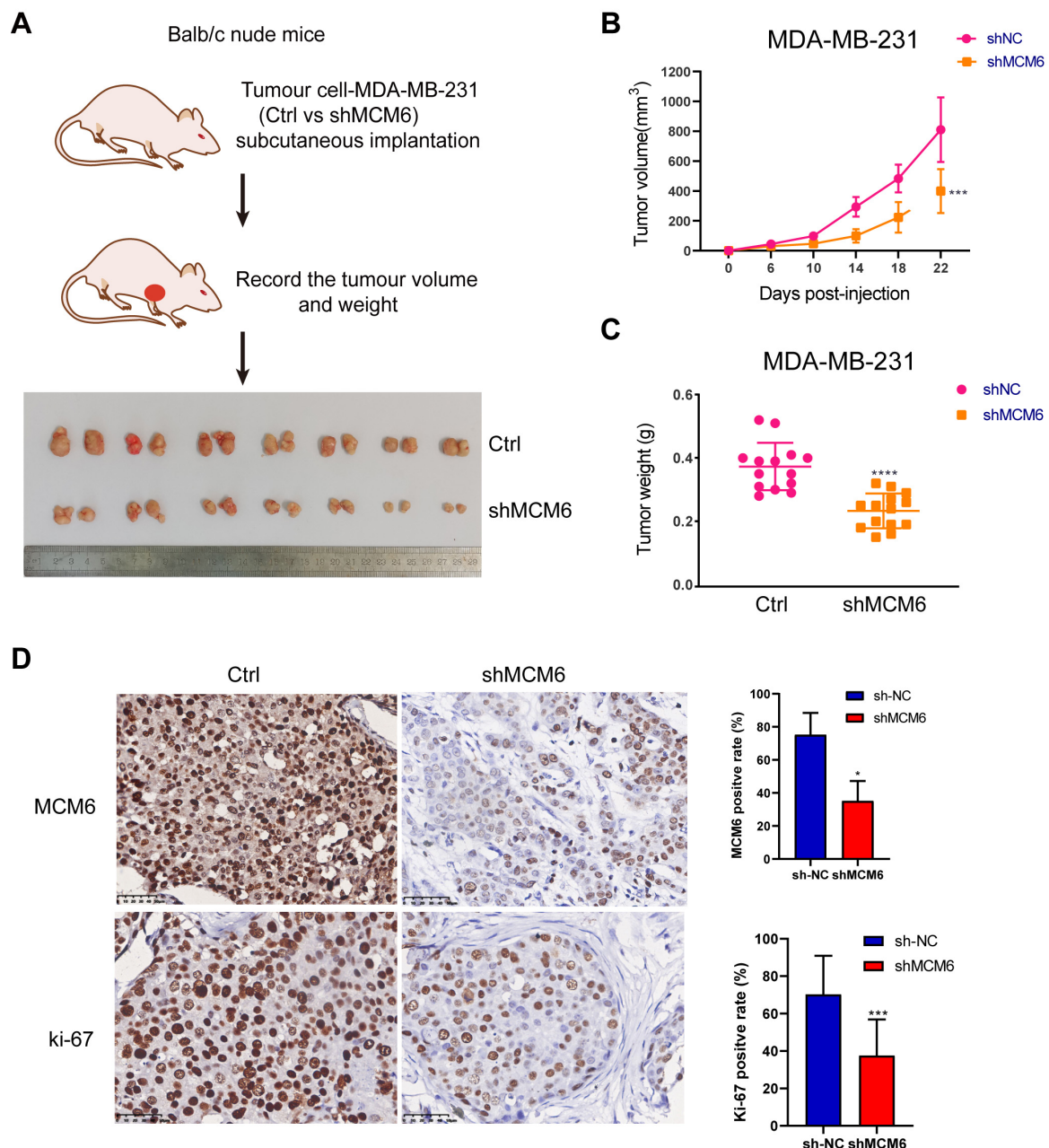


Fig. 10. CKAP4 promotes breast cancer cell growth *in vivo*. (A) Schematic diagram of the nude mouse xenograft model and all the tumors. (B,C) Knock down MCM6 significantly inhibits the growth of transplanted tumor in nude mice. (D) The immunohistochemical results showed that the expression of MCM6 was significantly decreased in the knockdown group, and the positive rate of Ki67 in the knockdown group was significantly lower than that in the control group. Statistics are presented as the means $\pm$ SDs. * $p < 0.05$, *** $p < 0.001$; **** $p < 0.0001$.

drug; PARP inhibitors iniparib, veliparib and olaparib for BRCA1/2 mutation, etc., has significantly prolonged life expectancy and quality of life in patients [26–28]. Although the mentioned drugs have achieved good clinical efficacy, they are only targeted at patients with specific molecular changes. There are still many breast cancer patients with poor efficacy because of drug resistance and other problems. Breast cancer is a very heterogeneous tumor, and its occurrence and development involve many gene changes.

Hence, it is imminent to explore new biological markers and potential targets of breast cancer to provide a new basis for prognostic effect management and the development of targeted medication.

This research suggested that MCM6 expression was increased in a variety of tumors, including breast cancer, combined with TCGA database, clinical samples and breast cancer cells. Our conclusions are supported by Issac *et al.* [16]. Furthermore, MCM6 expression in luminal B, basal

and HER2 breast cancer was significantly increased, and that in invasive lobular carcinoma was higher than that in invasive ductal carcinoma, which indicated that the role and prognostic value of MCM6 in different types of breast cancer may be different and that the mechanism needs to be further investigated.

In recent years, studies on biomarkers, prognostic markers and prognostic models of breast cancer have attracted increasing attention and can provide a good basis for the prognosis of breast cancer. We constructed a nomogram prediction model that included five factors (age, expression of ER, M stage, N stage and expression of MCM6) by multivariate Cox regression analysis, and the research results showed that low expression of MCM6 may indicate longer survival time for patients with breast cancer in our prognostic model. Relevant studies reported that excessive expression of MCM6 was related to poor prognostic effect. High expression of MCM6 mRNA was significantly associated with shorter survival time of patients with breast cancer [29]. Low expression of MCM6 mRNA is associated with increased recurrence-free survival in breast cancer patients [16]. The expression level of MCM6 in high-grade tumors is significantly increased and correlated with mitotic count, which is an effective reference index for tumor grading [30]. These results agreed closely with the outcomes in the model we constructed and suggested that overexpression was independent as a prognostic factor for breast carcinoma.

An increasing number of studies have shown that the role of MCM6 in tumor progression is extensive [14]. MCM6 silencing leads to arrest of S to G1 phase progression by reducing CDK2, CDK2, cyclin A, cyclin B4, cyclin D1 and cyclin E in hepatocellular carcinoma [31]. MCM6 promotes the metastasis of hepatocellular carcinoma through the MEK/ERK pathway [21]. CDK5RAP3 reduces MCM6 nuclear translocation, thereby reducing gastric cancer proliferation [32]. Liu *et al.* [33] found that SIX1 is an oncoprotein in cervical cancer that promotes proliferation by promoting the expression of MCM6. Boulay *et al.* [34] found that ARF1 promotes breast cancer proliferation by enhancing the expression of MCM6 and up-regulating the activity of pRB/E2F1. Nevertheless, the function of MCM6 in breast carcinoma remains unclear. The results of this study showed that MCM6 promoted breast cancer cell proliferation *in vitro* and *in vivo*, and promoted breast cancer migration and invasion *in vitro*. However, the biological pathway by which MCM6 plays a role in breast cancer remains unknown. By using GSEA, the study clarified the fact that overexpression of MCM6 promotes tumor progression mainly through the JAK-STAT3, mTOR myc, PI3K-AKT ERBB and p53 signaling pathways, which are closely related to tumorigenesis. Liu *et al.* [21] reported that MCM6 activates the MEK-ERK signaling pathway to promote the metastasis of liver cancer. We found that MCM6 overexpression is also related to the reg-

ulation of ubiquitination proteolysis, and whether MCM6 is involved in these signaling pathways has not been reported. Notably, MCM6 is involved in mismatch repair, which is consistent with the findings of Sherman *et al.* [35] reported that MCM6 was an important regulatory factor in the DNA replication system through complex formation with other MCM6 proteins to regulate DNA replication of the cell cycle in the carcinogenic process. In addition, our results indicated that the increased MCM6 expression had a bearing on cytotoxic effects of B-cell and T-cell receptor pathways and natural killer cells. Recent studies have shown that MCM6 functions to obstruct immune progression by reducing the immune response in microglial cells [14]. Therefore, MCM6 may be the key mediator of the immune response. We analyzed the relationship between MCM6 expression and immune checkpoint-related genes and immune cells. The results demonstrated that MCM6 displayed positive relevance with many immune checkpoint-related genes and negative correlation with BTNL9 in breast cancer, which has not been reported before and is worthy of further exploration. Whether MCM6 can affect the function of these immune checkpoints in breast cancer needs further study.

Given that MCM6 is key to the progression of breast carcinoma, we further explored the correlation between MCM6 and breast cancer-related chemotherapy drugs for therapy. Two potential drugs, PD-0332991 and trametinib, which are more sensitive when MCM6 is highly expressed, were screened out from breast cancer-related chemotherapy drugs. PD-0332991 is an inhibitor of CDK4 and CDK6 with oral activity and has antiproliferative activity on cancer cells, inducing their cell cycle arrest [36]. In addition, PD-0332991 guided the apoptosis of diffuse large B-cell lymphoma cells in a dose-dependent manner [37]. Studies have shown that PD-0332991 can be used for hormone (ER/PR)-positive and HER2-negative breast cancer [38]. Trametinib is an effective reversibility inhibitor of MEK 1/2 that restrains cell proliferation by blocking the MAPK pathway [39]. The FDA has approved trametinib combined with dabrafenib for patients with metastatic melanoma who have the BRAF V600E/K mutation [40]. Our results indicated that keener sensitivity to PD-0332991 and trametinib can be found in patients with overexpression of MCM6, which can be used as a monitoring indicator of drug sensitivity in breast cancer. However, our screening results still need to be verified by a large number of clinical cases and related experiments.

5. Conclusions

In conclusion, our research shows that overexpression of MCM6 can be found in patients with breast carcinoma and used as a poor prognostic factor for the disease. MCM6 can promote the proliferation of cancerous cells *in vitro* and *in vivo*. Based on database and bioinformatics analyses, MCM6 plays a crucial role in tumor immunity, tumor mu-

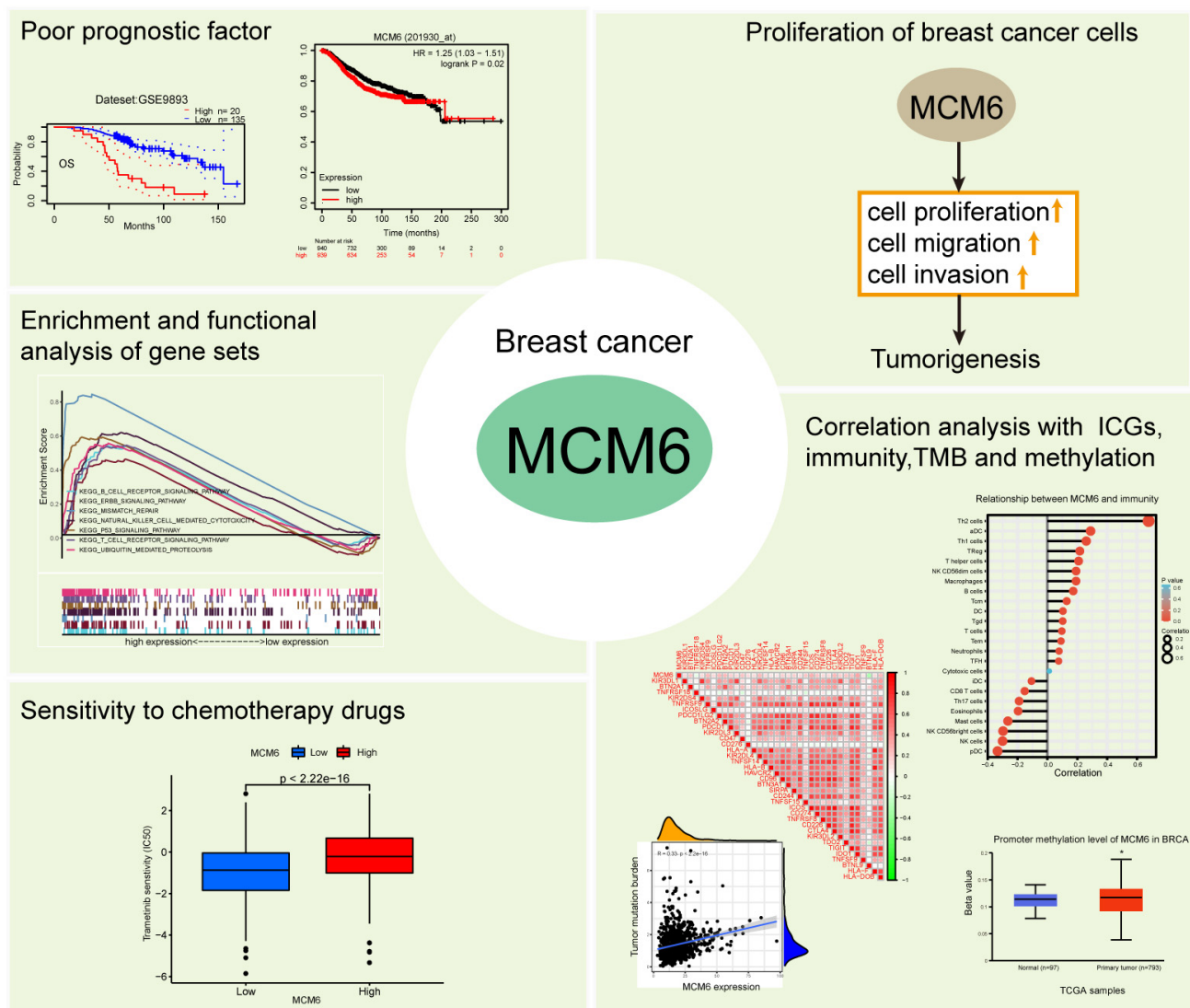


Fig. 11. MCM6 is a poor prognostic biomarker and promotes progression in breast cancer. Overexpression of MCM6 is poor prognostic biomarker in breast cancer. MCM6 can promote the proliferation of cancerous cells *in vitro* and *in vivo*. Based on database and bioinformatics analyses, MCM6 plays a crucial role in tumor immunity, tumor mutation burden and methylation through a variety of biological pathways and is related to drug sensitivity.

tation burden and methylation through a variety of biological pathways and is related to drug sensitivity (Fig. 11). MCM6 can be used as a poor prognostic marker of breast cancer and a potential target for immunotherapy and targeted therapy. However, in our experiments, we have not further verified the biological pathways of MCM6 in breast cancer and the role and mechanism of MCM6 in tumor mutation burden and tumor immunity, and further studies are needed. In the future, comprehensive individualized treatment with chemotherapy combined with targeted therapy and immunotherapy will become the main treatment option for breast cancer patients. Our preliminary study showed that the high expression of MCM6 in breast cancer increases the sensitivity of related drugs. Targeting MCM6 and combining with related sensitive drugs is a promising research

direction. Therefore, our study demonstrates that MCM6 has a potential oncogenic role in breast cancer and provides a new direction for targeted therapy and immunotherapy in breast cancer patients.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

ZL and GP designed the study. PW, DJ, and LL analyzed the data. ZL, YW, and YY performed the experiments. ZL, PW, and GP contributed to the writing of the manuscript. All authors contributed to editorial changes

in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work

Ethics Approval and Consent to Participate

This study was supported by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (kmmu20220767). Specimens were handled in accordance with legal and ethical regulations.

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Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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