Original Article

Combined targeting of KRT23 and NCCRP1 as a potential novel therapeutic approach for the treatment of triple-negative breast cancer

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Background: Breast cancers characterized by triple-negative status tend to be more malignant and have a poorer prognosis. A risk model for predicting breast cancer risk should be developed.

Methods: We obtained gene expression and clinical characteristics data using the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and The Cancer Genome Atlas (TCGA) database. Differential gene screening between patients with triple-negative breast cancer (TNBC) and non-triple-negative breast cancers (NTNBC) was performed according to the "edgeR" filter criteria. Univariate and multivariate Cox regression analyses were used to construct a risk model and identify prognosis-related genes. XCELL, TIMER, EPIC, QUANTISEQ, MCPCOUNTER, EPIC, CIBERSORT-ABS, and CIBERSORT software programs were used to determine the extent of tumor immune cell infiltration. To evaluate the clinical responses to breast cancer treatment, the half maximal inhibitory concentration (IC50s) of common chemotherapeutics were calculated using "pRRophetic" and "ggplot2". Cell proliferation was assayed using cell counting kit-8 (CCK8) and 5-Ethynyl-2′-deoxyuridine (EdU) Cell Proliferation Kit. A dual-luciferase reporter assay confirmed the gene regulatory relationship of sex determining region Y-box 10 (SOX10).

Results: An assessment model was established for Keratin23 (KRT23) and non-specific cytotoxic cell receptor 1 (NCCRP1) using the univariate and multivariate Cox regression analyses. In addition, high expression levels of KRT23 and NCCRP1 indicated high proliferation and poor prognosis. We also found that the gene expression patterns of multiple genes were significantly more predictive of risks and have a higher level of consistency when assessing risk. *In vitro* experiments showed that the expressions of KRT23 and NCCRP1 were increased in TNBCs and promoted cell proliferation. Mechanically, the dual-luciferase reporter assay confirmed that SOX10 regulated the expressions of KRT23 and NCCRP1. The risk score model revealed a close relationship between the expressions of KRT23 and NCCRP1, the tumor immune microenvironment, and chemotherapeutics.

Conclusions: In conclusion, we constructed a risk assessment model to predict the risk of TNBC patients, which acted as a potential predictor for chemosensitivity.

Keywords: Triple-negative breast cancers; Keratin23 (KRT23); non-specific cytotoxic cell receptor 1 (NCCRP1); tumor immune microenvironment

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Introduction

Breast cancer with triple-negative receptors lacks the genes for the estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2)/neu. It accounts for 15–20% of all breast cancers and has a poor prognosis (1,2). In the absence of ER, PR, and HER2/neu, optimizing the therapeutic management of patients is difficult (2,3). Despite the improvements in combined surgical and radiochemotherapy (4-6), there remains a pressing need for new therapeutic approaches and molecular targets.

Keratin23 (KRT23) belongs to the acidic type I keratins; it is strongly expressed in colon adenocarcinomas but absent in normal colon mucosa. KRT23 knockdown decreases proliferation and affects the DNA damage response of colon cancer cells (7). Similarly, researchers have found that phosphoprotein KRT23 accumulates in microsatellite-stable (MSS) colon cancers *in vivo* and impacts the viability and proliferation *in vitro* (8). However, KRT23 has not been reported in triple-negative breast cancers (TNBC). Non-specific cytotoxic cell receptor 1 (NCCRP1) is a paralog of the F-box superfamily of proteins (ubiquitin ligases) that regulate the cell cycle (9,10). In particular, NCCRP1 can act as a prognostic signature in pancreatic cancer and squamous cell carcinoma (11,12). However, the role of NCCRP1 in TNBC is unknown.

In this study, we first assessed the roles of KRT23 and NCCRP1 in predicting the diagnosis and prognosis of TNBC using bioinformatics analysis based on the Clinical Proteomic Tumor Analysis Consortium (CPTAC). We then validated the messenger RNA (mRNA) and protein expressions of KRT23 and NCCRP1 in breast cancer. In addition to elucidating the molecular mechanisms regulating the biological actions of KRT23 and NCCRP1 in a breast cancer cell line, the purpose of the study was to investigate the effects of KRT23 and NCCRP1 on the proliferation of breast cancer cells. We present the following article in accordance with the MDAR reporting checklist (available at https://gs.amegroups.com/article/view/10.21037/gs-22-486/rc).

Methods

Data retrieval and determination of the prognostic value

We obtained data on gene expression and clinical characteristics using the Clinical Proteomic Tumor Analysis Consortium (CPTAC) (https://cptac-data-portal.georgetown.edu/cptac) and The Cancer Genome Atlas (TCGA) database (https://tcga-data.nci.nih.gov/tcga). Differential gene screening between TNBC and non-triple-negative breast cancers (NTNBC) was performed according to the "edgeR" filter criteria (log2|fold change| >0.5, false discovery rate (FDR) <0.05). We then analyzed the obtained differential genes (Gene Ontology) GO and (Kyoto Encyclopedia of Genes and Genomes) KEGG enrichment pathways.

This study used univariate and multivariate Cox regression analyses to construct a risk model and identify prognosis-related genes. Our survival analysis was based on the Kaplan-Meier analysis (KM). XCELL (13), TIMER (14), EPIC (15), QUANTISEQ (16), MCPCOUNTER (17), CIBERSORT-ABS (18), and CIBERSORT (18) software were used to determine the extent of tumor immune cell infiltration. To evaluate the clinical responses to breast cancer treatment, the half maximal inhibitory concentration (IC50s) of common chemotherapeutics were calculated using "pRRophetic" and "ggplot2".

Clinical specimens and approval

Breast cancer tissues and corresponding adjacent normal tissues were collected from 29 patients who underwent surgical resection at the First Affiliated Hospital of Gannan Medical University, China. After surgical removal, the samples were immediately frozen in liquid nitrogen. This study was conducted according to the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University (No. LLSC-2022033101). Informed consent was obtained from the patients or their guardians.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was extracted using an RNA Extraction Kit (Qiagen, USA) according to the manufacturer's instructions. Next, the RNA was reverse-transcribed using the TAKARA reverse transcription kit (TaKaRa, Dalian, China). TaKaRa SYBR Premix Ex Taq^{TM} II Kit (TaKaRa, Dalian, China) was used to amplify the complementary DNA (cDNA)templates by qRT-PCR. The ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA) was used to perform qRT-

PCR. The primer sequences are listed in Table S1.

Cell cultures and lentiviral transfection

Human breast cancer cells (MDA-MB-231 cell lines) were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). The MDA-MB-231 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Foetal Bovine Serum (FBS), 100 U/mL penicillin, and 100 mU/mL streptomycin (Corning, USA).

Overexpression of KRT23 and NCCRP1 (lv-KRT23 and lv-NCCRP1) were constructed and transfected in MDA-MB-231 cells using lentiviral overexpression plasmids Ubi-MCS-SV40-puromycin. Cells transfected with lv-negative control (NC) lentivirus served as a negative control. Next, we used the hU6-MCS-CMV-puromycin plasmid to knock down KRT23 and NCCRP1 in cells (sh-KRT23 and sh-NCCRP1) before transfection in MDA-MB-231 cells, with sh-NC cells used as a negative control. The culture was performed in 5% carbon dioxide (CO_2) incubators (Thermos, USA). The interfering nucleotide sequence was designed according to the Invitrogen RNA interference sequence design website (https://rnaidesigner.thermofisher.com/). The RNA interference target sequences are presented in Table S2.

Immunohistochemistry and western blot (WB) assays

Immunohistochemistry was performed according to the manufacturer's instructions (Solarbio, Beijing, China). Sections were observed, and images were captured under a Leica DMRB microscope (Leica Microsystems, Germany). An extraction kit for total proteins (Keygen, Nanjing, China) was used to extract proteins from cells. Protein concentrations were determined using the BCA-100 Protein Quantitative Analysis Kit (Shanghai, China). WB was performed according to the standard protocols, and WB analysis was conducted using the antibodies listed in Table S3.

Cell Counting Kit 8 (CCK8) and 5-Ethynyl-2'-deoxyuridine (EdU) Cell Proliferation Kit

Briefly, MDA-MB-231 cells (1×10^3) were seeded into 96-well plates. At various time points (0, 1, 2, 3, and 4 days), we determined the value of absorbance (at 450 nm) for each cell according to the instruction manuals (Dojindo, Japan). To conduct the EdU assay, we followed the instructions provided with the EdU Cell Proliferation Assay Kit (RiboBio Co., Ltd., Guangzhou, Guangdong, China). Finally, the EdU-stained cells were examined under a confocal microscope (Carl Zeiss, Germany).

Dual-luciferase reporter assay

To explore the effect of SOX10 on the KRT23 and NCCRP1 promoter activity, the sequences of the KRT23 and NCCRP1 promoters were sub-cloned downstream of the luciferase reporter gene to create pGL3-KRT23 pro-Wild-type (Wt) and pGL3-NCCRP1 pro-Wild-type (Wt) plasmids. To test the binding specificity, a corresponding mutant was created with a changed region binding site to create pGL3-KRT23 pro-Mut-type (Mut) and pGL3-NCCRP1 pro-Mut-type (Mut) plasmids. The sequences of the wild-type KRT23 and NCCRP1 promoters were obtained from the UCSC genome browser (http://genome.ucsc.edu/). Luciferase activity was measured using a luciferase assay kit (K801–200, BioVision Inc., Milpitas, CA, USA). The relative luciferase activity was calculated based on firefly fluorescence versus Renilla fluorescence.

Statistical analysis

All statistical analyses were performed by using R software packages version 3.4.2. Experimental data analysis was performed using GraphPad Prism 8, with $P<0.05$ considered statistically significant.

Results

Identification of potential key genes associated with the prognosis of TNBC

We determined the differential expression of proteins between TNBC and NTNBC based on the CPTAC database (available at https://cdn.amegroups.cn/static/public/gs-22-486-1.xlsx). Next, we analyzed the differential proteins by GO and KEGG enrichment pathways. GO enrichment analysis revealed five significantly enriched GO terms (carboxylic acid biosynthetic process, gland development, gland morphogenesis, organic acid biosynthetic, and urogenital system development) ($P<0.05$) (*Figure 1A*). The KEGG enrichment analysis identified five significantly enriched KEGG pathways (arginine biosynthesis, estrogen signaling pathway, fatty acid

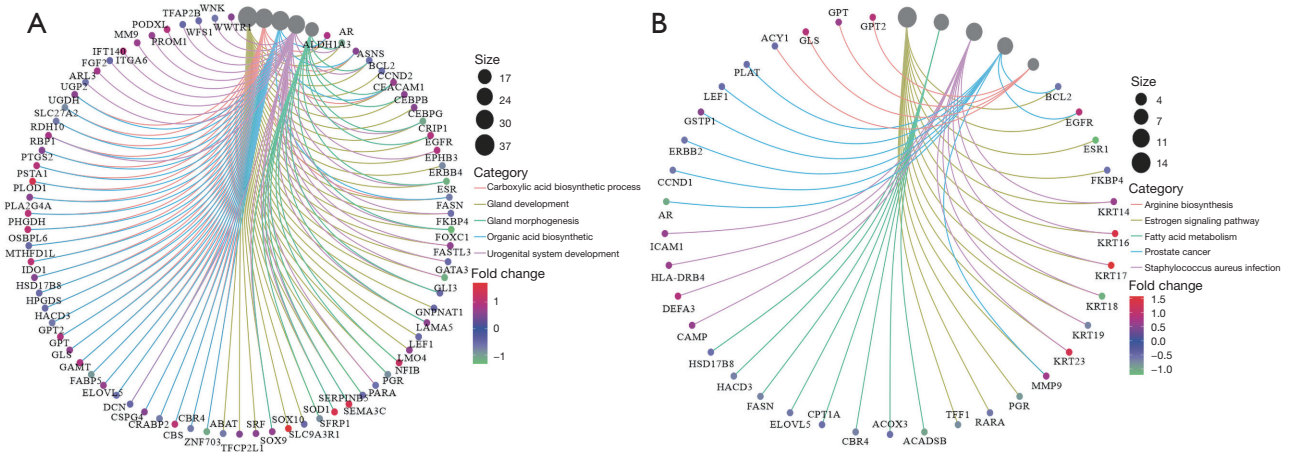

Figure 1 GO and KEGG pathway enrichment analysis of target genes. (A) GO enrichment analysis. (B) KEGG enrichment analysis. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

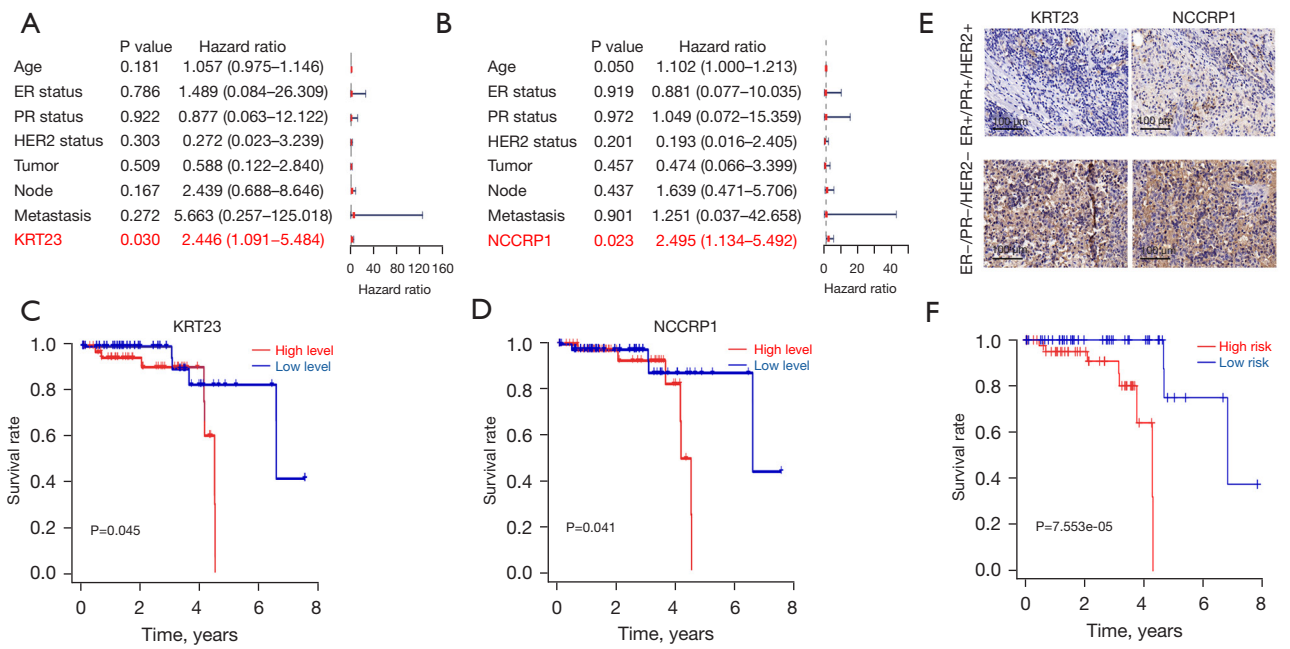

Figure 2 Identification of potential key genes associated with the prognosis of triple-negative breast cancer. (A,B) Multivariate analysis of prognostic factors. (C) The overall survival curves of breast cancer patients with high and low KRT23 levels. (D) The overall survival curves of breast cancer patients with high and low NCCRP1 levels. (E) Immunohistochemical findings of KRT23 and NCCRP1 expression in breast cancer tissues. Bar =100 μm. (F) Survival curves of patients in the high- and low-risk groups. ER, estrogen receptor; PR, progesterone receptor; HER2, human epidermal growth factor receptor 2.

metabolism, prostate cancer, and staphylococcus aureus infection) (P<0.05) (*Figure 1B*).

All differential proteins were analyzed to identify prognosis-related genes in triple-negative breast cancer. The univariate and multivariate Cox regression analyses found that KRT23 and NCCRP1 could independently predict prognosis (*Figure 2A,2B*), with high KRT23 and NCCRP1 expression levels indicating poor prognosis in triple-negative breast cancer (*Figure 2C,2D*). Immunohistochemistry showed that KRT23 and NCCRP1

had a high expression in TNBC tissues (*Figure 2E*).

Next, the risk score was calculated for each of the 105 patients based on the KRT23 and NCCRP1 expression levels from CPTAC, and the patients were then divided into high- and low-risk groups based on a cutoff value (the median risk score). An analysis of the Kaplan-Meier survival data revealed that the high-risk group had worse overall survival (OS), which suggests that the risks may have a prognostic value (*Figure 2F*).

High KRT23 and NCCRP1 protein levels promote breast cancer cell proliferation

To further explore the biological role of KRT23 and NCCRP1 in breast cancer cells, the expressions of KRT23 and NCCRP1 were knocked down or overexpressed in MDA-MB231 cells using lentivirus (*Figure 3A*,*3B*). The EdU and CCK-8 assays showed that silenced KRT23 or NCCRP1 restored cell proliferation (*Figure 3C-3E*). However, the overexpression of KRT23 or NCCRP1 promoted cell proliferation (*Figure 3F-3H*). These results suggested that KRT23 or NCCRP1 might be essential oncogenes in TNBC.

SOX10 regulates KRT23 and NCCRP1 gene expression in MDA-MB231 cells

The Venn diagram analysis identified the differentially expressed transcription factors, which co-regulated KRT23 or NCCRP1 expression in TNBC. The results showed that KRT23 and NCCRP1 had 12 differentially expressed common transcription factors (*Figure 4A*). An overall correlation between the 14 features is displayed in a heatmap, and a significant correlation was found between SOX10 expression and KRT23 or NCCRP1 expression (*Figure 4B*).

Additionally, we found that SOX10 was positively correlated with KRT23 and NCCRP1 in TNBC tissues (*Figure 4C*,*4D*). Meanwhile, KRT23 and NCCRP1 expression was promoted by SOX10 overexpression (*Figure 4E*,*4F*). Dual-luciferase reporter assays in MDA-MB231 cells were performed to validate the regulation of KRT23 and NCCRP1 expression by SOX10 (*Figure 4G*).

The risk score was associated with immune cell infiltration and chemotherapeutics

In this study, multiple software programs (XCEL, TIMER, QUANTISEQ, MCPCOUNTER, EPIC, CIBERSORT-ABS, CIBERSORT) were used to analyze the relationships between patients in the high- and low-risk groups and immune cells in the risk prediction model (*Figure 5A*). To determine whether the chemotherapeutic treatment efficacy was correlated with the risk scores, we examined the relationship between risk scores and IC50.

In the present study, we sought to determine the association between the risk score and the efficacy of common chemotherapeutic agents in the treatment of breast cancer in TCGA database. Our results revealed that a high-risk score was related to a lower half maximal inhibitory concentration (IC50) of chemotherapeutics such as Sorafenib (*Figure 5B*) and Lapatinib (*Figure 5C*), while a low-risk score was related to a lower IC50 of chemotherapeutics such as Bleomycin (*Figure 5D*), Embelin (*Figure 5E*), Tipifarnib (*Figure 5F*), and Temsirolimus (*Figure 5G*). These results indicated that the model was a potential predictor of chemosensitivity.

Discussion

Breast cancers characterized by triple-negative status tend to be more malignant and have a poorer prognosis (1,2,4-6,19,20). To decrease the mortality rate and improve the prognosis of breast cancer patients, a model for predicting breast cancer risk should be developed. In this study, a risk model was constructed and validated using the CPTAC databases.

Using multivariate and univariate Cox regression analyses, an assessment model was established for KRT23 and NCCRP1. We found that high expression levels of KRT23 and NCCRP1 were indicative of poor prognosis and that the gene expression patterns of multiple genes are considerably more predictive of future risks and have a higher level of consistency when assessing risk. KRT23 is a newly discovered member of the keratin family. Various tumor tissues, including pancreatic cancer (21), colorectal carcinoma (7,22-24), and hepatocellular carcinoma (25,26), have exhibited aberrant expression of KRT23. Our findings support the same results, namely that the expression of KRT23 was increased in TNBC and promoted cell proliferation. In this study, we investigated the expression and functions of NCCRP1 in cells. Our findings also found the same; the expression of NCCRP1 was increased in TNBC and promoted cell proliferation. Therefore, we can design specific antibodies against KRT23 and NCCRP1 to block their effects on breast cancer proliferation.

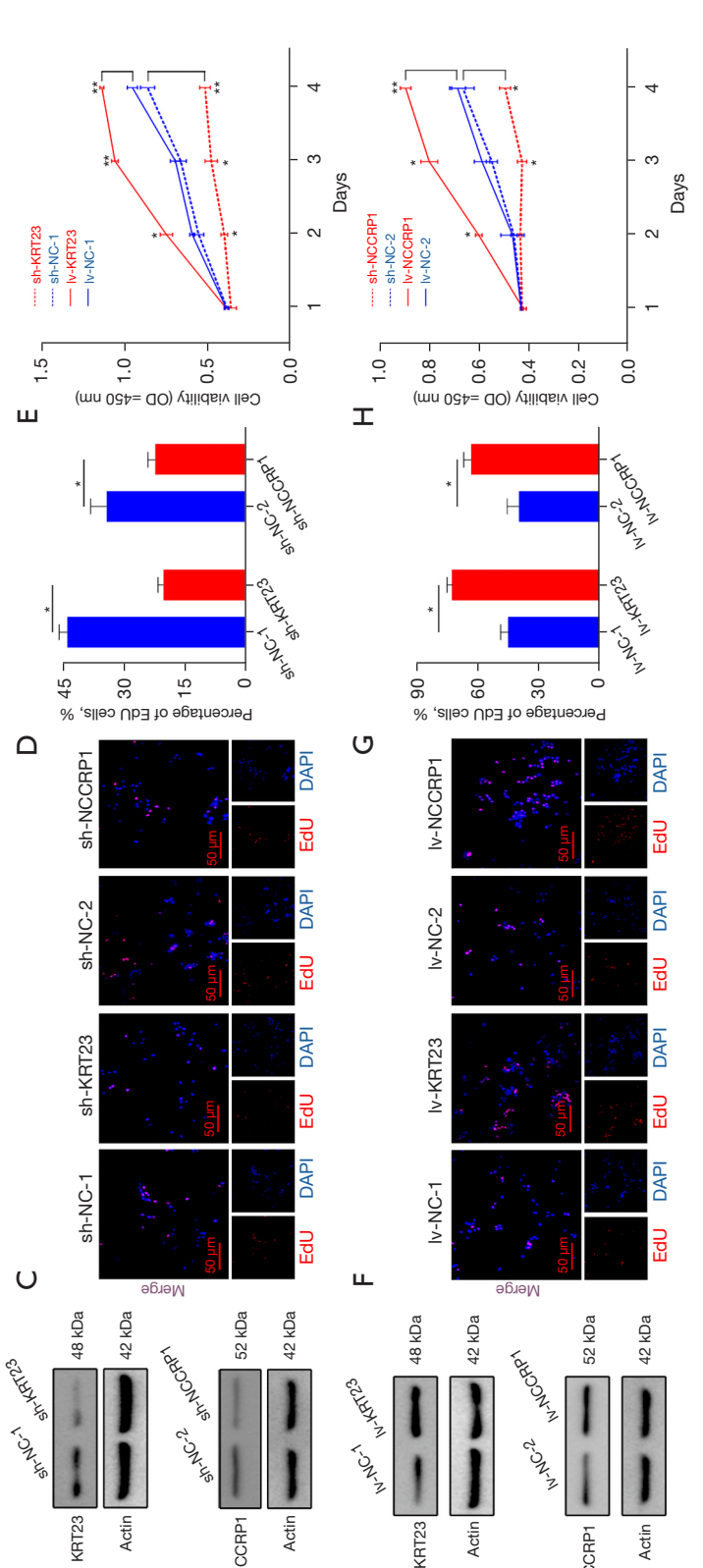

Figure 3 High KRT23 and NCCRP1 protein levels promote breast cancer cell proliferation. (A) Knockdown efficiency of KRT23 and NCCRP1 was examined by WB analysis. (B) Overexpression efficiency of KRT23 and NCCRP1 was examined by WB analysis. (C,D) Detection of the proliferation of cells after interference with KRT23 or NCCRP1 by EdU; *, represents P<0.05. (E) Detection of the proliferation of cells after interference with KRT23 or NCCRP1 by CCK8 assay; *, represents P<0.05, **, represents P<0.01. (F,G) Detection of the proliferation of cells after overexpression with KRT23 or NCCRP1 by EdU; *, represents P<0.05. (H) Detection of the proliferation of cells after overexpression with KRT23 or NCCRP1 by CCK8 assay; *, represents P<0.05, **, represents P<0.01. DAPI, Fluorescent DNA Stain; WB, Western Blot; EdU, Ethynyl-2′-deoxyuridine; OD, Optical density; NC, Negative control.

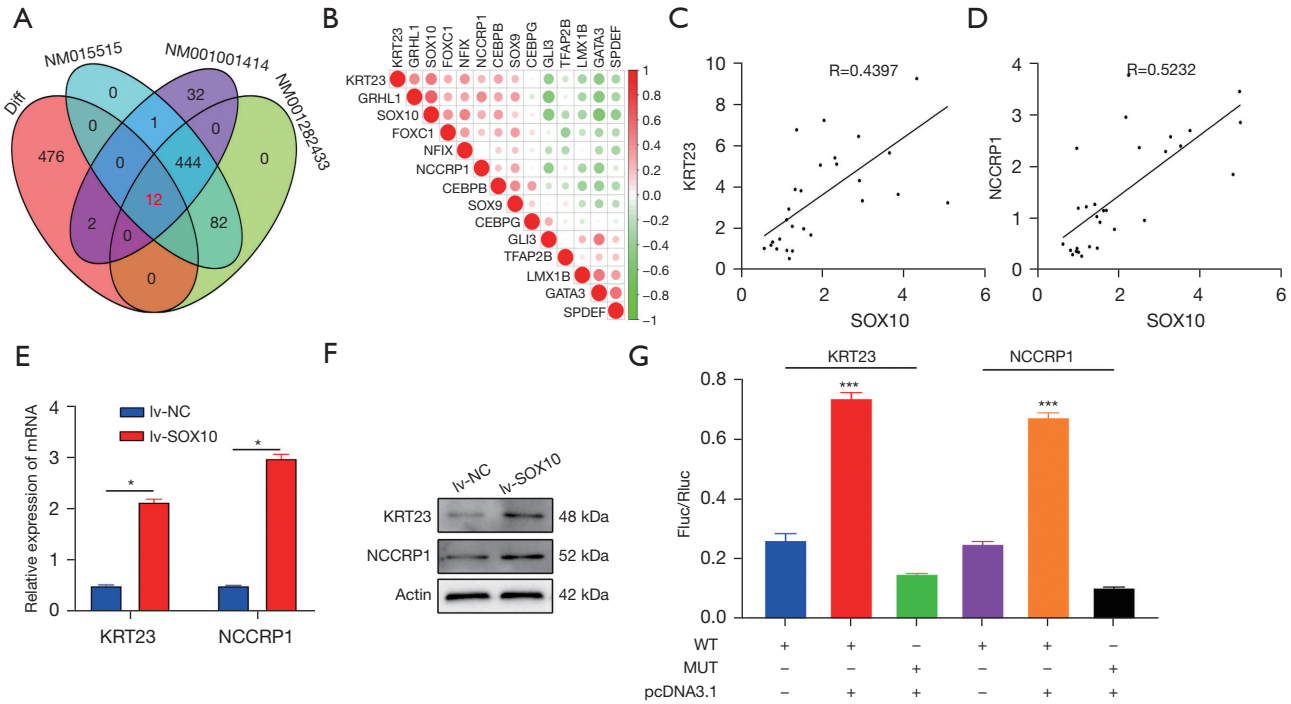

Figure 4 SOX10 regulates KRT23 and NCCRP1 gene expression in MDA-MB231 cells. (A) Venn diagram analysis identified the differentially expressed transcription factors in triple-negative breast cancer. (B) Heatmap of the correlation analysis of transcription factors and KRT23 and NCCRP1. (C) Correlation between the SOX10 and KRT23 mRNA expression in triple-negative breast cancer. (D) Correlation between the SOX10 and NCCRP1 mRNA expression in triple-negative breast cancer. (E,F) The gene and protein expressions of KRT23 and NCCRP1 in the overexpressed SOX10 cells was explored by qRT-PCR or WB; *, represents P<0.05. (G) Analysis of KRT23 and NCCRP1 promoter activity by dual-luciferase reporter assay; ***, represents P<0.001. WT, wild-type; MUT, mut-type; qRT-PCR, quantitative real-time polymerase chain reaction; WB, western blot.

To identify the transcription factors regulating the joint regulation of KRT23 and NCCRP1, we found their transcripts through the UCSC database and performed transcription factor prediction on all transcripts. Venn diagram analysis identified 12 differentially expressed transcription factors, which co-regulated KRT23 or NCCRP1 expression in TNBC. We confirmed our hypothesis that SOX10 regulated KRT23 and NCCRP1.

We also analyzed the risk scores associated with immune cell infiltration and chemotherapeutics. The XCEL database showed a strong association with the common lymphoid progenitor, M2 macrophages, and $CD4^+$ Th1 T cells in high-risk group patients. The TIMER database showed a strong association with macrophages in high-risk group patients. The QUANTISEQ database demonstrated a strong association with uncharacterized cells in high-risk group patients. The EPIC database showed a strong association with cancer-associated fibroblasts in high-risk group patients. The CIBERSORT-ABS database showed a strong association with neutrophils in high-risk group patients. The CIBERSORT database showed a strong association with M2 macrophages and neutrophils in high-risk group patients. Based on these results, we found that high-risk patients were closely associated with M2 type macrophage infiltration. Therefore, in the treatment of triple negative breast cancer, it can be combined with M2 macrophage inhibitor to improve the efficacy of chemotherapy. Similarly, we can also predict the relationship between KRT23 and NCCRP1 and immune cells by analyzing the correlation between KRT23 and NCCRP1 and immune-related genes.

The sensitivity to chemotherapy was different between

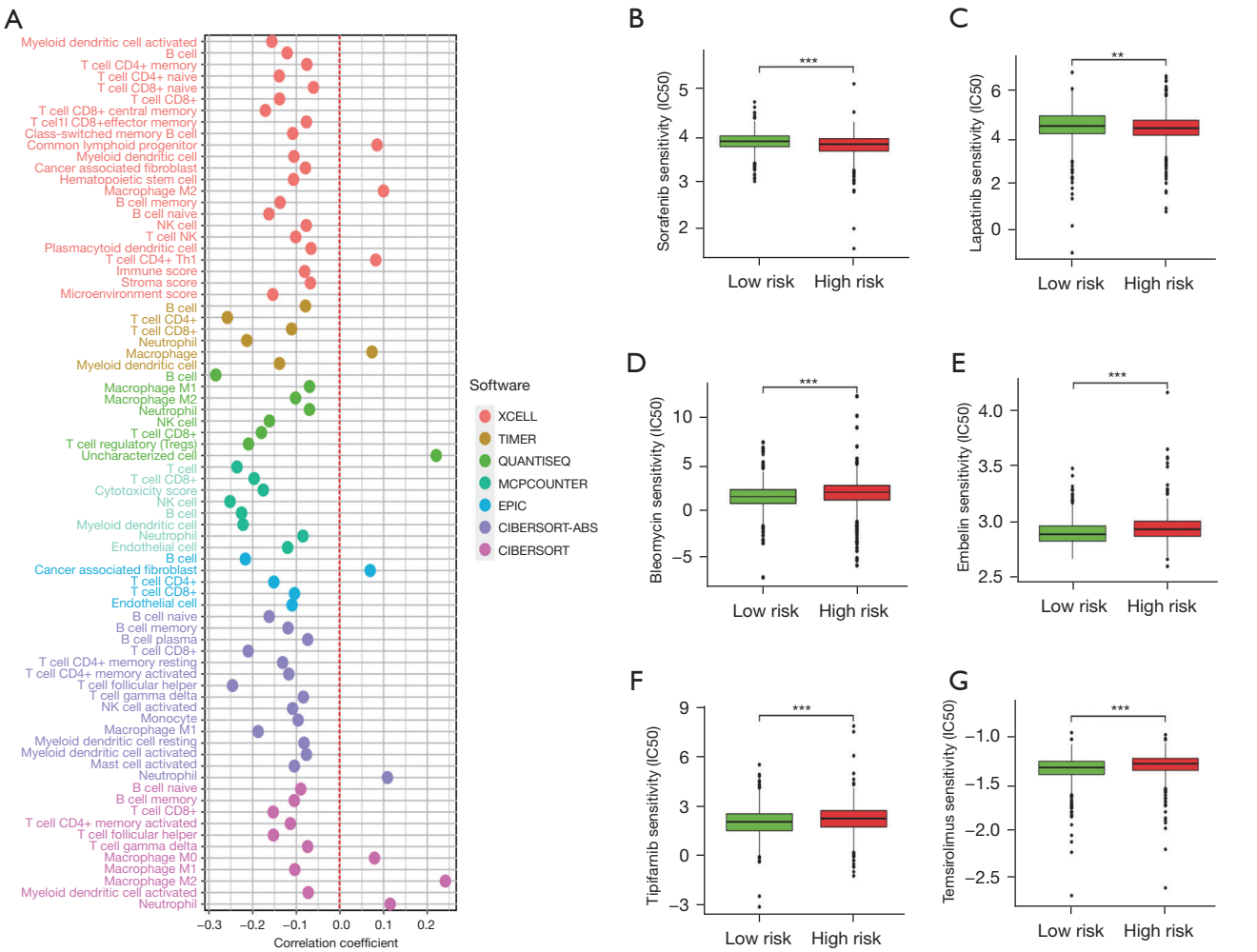

Figure 5 The risk score was associated with immune cell infiltration and chemotherapeutics. (A) Summary of the abundances of 22 types of immune cells, as estimated using the XCELL, TIMER, QUANTISEQ, MCPCOUNTER, EPIC, CIBERSORT-ABS, and CIBERSORT analytical tools for different risk groups. (B-G) The half maximal inhibitory concentration (IC50) of some chemotherapeutic drugs commonly used to treat breast cancer, using the "pRRophetic" R package; **, represents P<0.01, *** represents P<0.001.

the high- and low-risk groups. In conclusion, these results indicated that the model was a potential predictor of chemosensitivity.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://gs.amegroups.com/article/view/10.21037/gs-22-486/rc

Data Sharing Statement: Available at https://gs.amegroups.com/article/view/10.21037/gs-22-486/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://gs.amegroups.com/article/view/10.21037/gs-22-486/coif). The authors have no conflicts of interest to declare

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related

to the accuracy or integrity of any part of the work are appropriately investigated and resolved. This study was conducted according to the Declaration of Helsinki (as revised in 2013) and was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical University (No. LLSC-2022033101). Informed consent was obtained from the patients or their guardians.

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Supplementary

Table S1 Primers for quantitative real-time PCR

Genes	Forward primers (5'-> 3')	Reverse primers (5'-> 3')
NCCRP1	ATTTCCGTGGCTGGTACATTAG	ATGGCTGGTTGTTCGTCATCC
KRT23	TACTAGGCGGAAATGGGAAGG	TCTTACCATCCACTATCTGCTCC
SOX10	CCTCACAGATCGCCTACACC	CATATAGGAGAAGGCCGAGTAGA
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

PCR, polymerase chain reaction.

Table S2 Target sequences of shRNAs used for gene knockdown in breast cancer cells

shRNA	Sequence (5'-3')
NCCRP1-shRNA	GCAATTTCCGTGGCTGGTACA
KRT23-shRNA	GGCCAACATGAAGCTGGAAAG

Table S3 Information on antibodies used in breast cancer

Reagent	Source	Identifier
Anti-NCCRP1	Novus Biologicals	Cat#NBP2-13642/NBP1-57053
Anti-KRT23	Abcam	Cat#ab156569
Anti-Actin	Abcam	Cat#ab8226

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