Original paper

Clinical significance and effect of PRR11 up-regulation on the malignancy of osteosarcoma

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Osteosarcoma ranks first in both morbidity and mortality among primary bone tumors. This study aimed to investigate the effect of up-regulation of PRR11 on the malignancy of osteosarcoma and its clinical significance.
The expression, biological function, related pathways of PRR11 in osteosarcoma and its impact on prognosis were explored through the bioinformatics database. After PRR11 was up-regulated, cell proliferation, invasion, migration and apoptosis were detected by the cell counting kit-8 method, Transwell, scratch, and flow cytometry. PRR11 is highly expressed in a variety of malignant tumors, including osteosarcoma tissue and cells, and has a significant impact on prognosis. Univariate Cox regression analysis revealed that PRR11 was an independent prognostic factor for osteosarcoma. The gene set enrichment analysis results showed that the differential genes were mainly enriched in the biological process of the cell cycle; the protein-protein interaction network mainly interacted with the regulatory genes of the cell cycle. PRR11 promotes the invasion, migration, and proliferation of osteosarcoma cells and inhibits their apoptosis.
Comprehensive bioinformatics analysis revealed that PRR11 promotes the malignancy of osteosarcoma cells mainly by participating in cell cycle regulation, and has an important impact on osteosarcoma prognosis. PRR11 may provide the basis for prognosis and treatment in patients with osteosarcoma.

Key words: *PRR11*, osteosarcoma, bioinformatics, prognosis, cell cycle.

Introduction

Osteosarcoma is a malignant tumor of the skeletal system, which is derived from mesenchymal cells, and has the highest morbidity and mortality among all primary bone tumors [1]. Currently accepted treatments for osteosarcoma include surgery, chemotherapy, and radiation therapy [2]. With the continuous improvement in the treatment methods, the five-year survival rate of patients with osteosarcoma has reached 60–70%. However, the overall five-year survival of patients with recurrent or metastatic osteosarcoma has undergone little change and is approximately 20% [3]. In recent decades, with the maturation and wide application of second-generation high-throughput sequencing technology, extensive research on tumor tissue and cell sequencing has provided reliable data for gene-targeted therapy. Oncology research has thus been ushered into the era of "data explosion". Bioinformatic methods have been used to screen tumor mRNA [4], circRNAs [5], lncRNA [6], the circRNA-miRNA-mRNA network [7], tumor microenvironment genes [8], and N6-methyladenosine [9]. Although there have been several studies, the clinical outcomes of patients with osteosarcoma have not significantly improved; there-

fore, the identification of new effective treatments is critical.

PRR11, a newly identified gene with oncogenic potential, is a proline-rich protein encoded by the PRR11 gene located in the amplified region of 17q22 [10]. It has been found that PRR11 participates in cell cycle regulation by regulating cell cycle transcription factor-related binding sites, thus affecting cell proliferation, apoptosis, cell cycle progression, and carcinogenesis [11]. It was confirmed that PRR11 being expressed highly by various malignant tumors has a significant correlation with prognosis. However, the role of PRR11 in osteosarcoma has only been reported in a few studies [12]. At present, the treatment of osteosarcoma has hit a bottleneck. In this study, we used bioinformatics to mine public database data for analyzing the expression of PRR11 in osteosarcoma; further, we studied the clinicopathological characteristics of PRR11 and its relationship with osteosarcoma prognosis. Simultaneously, we verified the malignant biological behavior of PRR11 in osteosarcoma *via* in vitro cell experiments, which may help in determining a targeted therapy for osteosarcoma.

Material and methods

Public database data retrieval

In the Gene Expression Profiling Interactive Analysis (GEPIA) database, we searched the expression of the *PRR11* gene in various normal tissues and cancer tissues. In the Cancer Cell Line Encyclopedia (CCLE) database, we downloaded the *PRR11* transcriptome data and analyzed the differential expression level of PRR11 between osteosarcoma and other cancer cell lines. The whole transcriptome sequencing data and clinical data of *PRR11* in osteosarcoma tissues from the The Cancer Genome Atlas (TCGA) database were downloaded using UCSC Xena.

Data processing

We used the "ggplot2" package in the R software (4.1.2) to process the PRR11 transcriptome data downloaded in CCLE. Using the "DESeq2" package, the samples were chopped up into a high and a low expression group based on the median of PRR11 expression, and the whole transcriptome sequencing data of the *PRR11* gene in osteosarcoma tissues in TCGA were analyzed. The clinical data were analyzed by univariate Cox regression and Kaplan-Meier survival curve operating with the "survival" and "survminer" packages.

Gene set enrichment analysis and protein-protein interaction network analysis

The gene set enrichment analysis software was used to analyze the gene expression matrix processing of samples of 256 patients with osteosarcoma obtained from the TCGA database, and pathways with a p-value < 0.01 and false discovery rate < 0.25 were screened out. Using STRING to construct a protein-protein interaction (PPI) protein network with PRR11 protein as the core, the biological functions of these proteins were analyzed. It is significant for elucidating the mechanism by which PRR11 adjusts the occurrence and progression of osteosarcoma. Retrieval conditions: 1) Protein Name: PRR11; 2) Organism: *Homo sapiens*.

Cells and main reagents

The osteosarcoma cell line MG63 was sourced from the Shanghai Cell Bank, Chinese Academy of Sciences. PRR11 rabbit anti-human polyclonal antibody (Ab237526) was bought from the Abcam Company, USA. Trizol was bought from Ambion, USA. Reverse transcription kit PrimeScript II RTase and Recombinant RNase Inhibitor were bought from TAKARA. Goat anti-mouse IgG, goat anti-rabbit, and DAB chromogenic kits were bought from OriGene, USA. SYBR FAST qPCR Master Mix was bought from KAPABiosystems. Lipofectamine 2000 was bought from Invitrogen, USA. MEM, Opti-MEM, and fetal bovine serum were bought from Gibco, USA. PBS, 0.25% trypsin, and the cell counting kit were bought from Beijing solarbio. Transwell chambers were bought from Corning, USA.

Cell culture and transfection

MG63 cells were cultured in 10% MEM in an incubator under culture conditions of 5% CO_2, 10% humidity, and a temperature of 37°C. Transfection was performed using Lipofectamine 2000 cationic lipid transfection reagent based on the manufacturer's instructions. MG-63 cells in the log phase were taken and seeded in a 6-well plate at a density of 5×10^5 cells/well for cell culture. Lipofectamine 2000 was used to transfect the empty control group and the PRR11 high expression group. After four hours, the medium containing the transfection reagent was replaced with the normal medium. The transfected cells were cultured for 48 hours at 37°C and in a 5% CO_2 incubator, which will be used for subsequent experiments.

Quantitative real-time polymerase chain reaction

Trizol reagent was used to extract total RNA from the cells based on the kit instructions. cDNA was obtained by the reverse transcription kit, and the amplification of the target gene was obtained by the fluorescent quantitative PCR kit. The relative expression level of RNA was expressed by 2 -ΔΔCt. The experiments were repeated three times. See Table I for the primer sequences.

Table I. Primer sequences used for quantitative real-time polymerase chain reaction

Gene	Primer	SequeSiRNA-NCe (5′–3′)	PCR Products
Homo GAPDH	Forward	GGGAAACTGTGGCGTGAT	299 bp
	Reverse	GAGTGGGTGTCGCTGTTGA	
Homo PRR11	Forward	AACTTACAAATGTGCCTGCC	186 bp
	Reverse	CCTGAAGTGCTTTAGCGAGA	

Total protein extraction and western-blot detection

The total protein was extracted with RIPA lysate, and quantitatively evaluated by the BCA protein assay kit. Subsequently, 20 μg of protein solution per well was loaded for SDS-PAGE electrophoresis, and the proteins were transferred to a PVDF membrane, soaked in the primary antibody incubation solution, and incubated overnight at 4°C. The next day, the membrane was washed with PBST, then incubated with the secondary antibody for 1 h at room temperature, washed with PBST, followed by detection of light emission by control label. The membrane was placed in an automatic chemiluminescence analyzer and the gray value of the relevant bands was read by TANON GIS software. The data were independently replicated three times.

Cell proliferation assay

The cells that were stored in a 96-well plate were cultured in an incubator at 37°C. Ten μl of cell counting kit-8 solution was added at culture time intervals of 24, 48, and 72 hours, and after 1 hour incubation. We used a microplate reader to detect the absorbance value at 450 nm and draw the proliferation curve. The experiment was repeated three times.

Apoptosis detection

For apoptosis detection, 1×10^6 cells were digested into a cell suspension, then Annexin V-PE and 7-AAD were added, followed by incubation in the dark for 30 minutes at 4°C. On detection by flow cytometry, strong 7-AAD indicates dead cells, weak 7-AAD indicates apoptotic cells, and no 7-AAD indicates normal viable cells. The NovoExpress analysis software was used for calculating the number of apoptotic cells. The data were independently replicated three times.

Invasion assay

A 0.5 ml cell suspension containing a concentration of 1×10^5 cells/ml was inoculated into each transwell. Subsequently, 0.75 ml of 10% fetal bovine serum medium was added to the lower 24-well plate and incubated at 37°C for 24 hours. We stained the cells with crystal violet solution and observed the number of cells using a microscope.

Wound healing assay

A total of 1×10^6 cells were added to each 6-well plate. When the bottom of the 6-well plate was covered with cells, a sterilized pipette tip was used to draw three lines, which were parallel to the central axis. Photographs of the scratches were taken at 0 and 24 hours, and ImageJ software was used to measure the width of the scratches.

Statistical analysis

The data were analyzed by SPSS 22.0 statistical software, and measurement data were expressed as means ± standard deviation. The differences among multiple groups were compared by ANOVA. The statistical significance was set at $p < 0.05$.

Ethical statement

This study is mainly aimed at cell experiments and bioinformatics database research, and does not involve animal experiments and patient studies. This experiment has been solicited for ethical review by the Ethics Committee of the First Affiliated Hospital of Shihezi University School of Medicine (batch number: KJ2022-138-01). This study was exempt from ethical approval.

Data availability statement

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

Results

PRR11 expression in malignant tumors and tumor cells

In the GEPIA database, it was revealed that PRR11 was higher in most malignant tumors compared to normal tissues (Fig. 1A), and was higher in sarcomas, with the difference being significant ($p < 0.05$) (Fig. 1B). In order to understand the expression of PRR11 in osteosarcoma cells, data from the CCLE database were analyzed and the results showed that the expression of PRR11 in osteosarcoma cells ranked eighth among the 33 malignant tumor cell lines included in it (Fig. 1C). There were also differences in the expression of PRR11 among the osteosarcoma

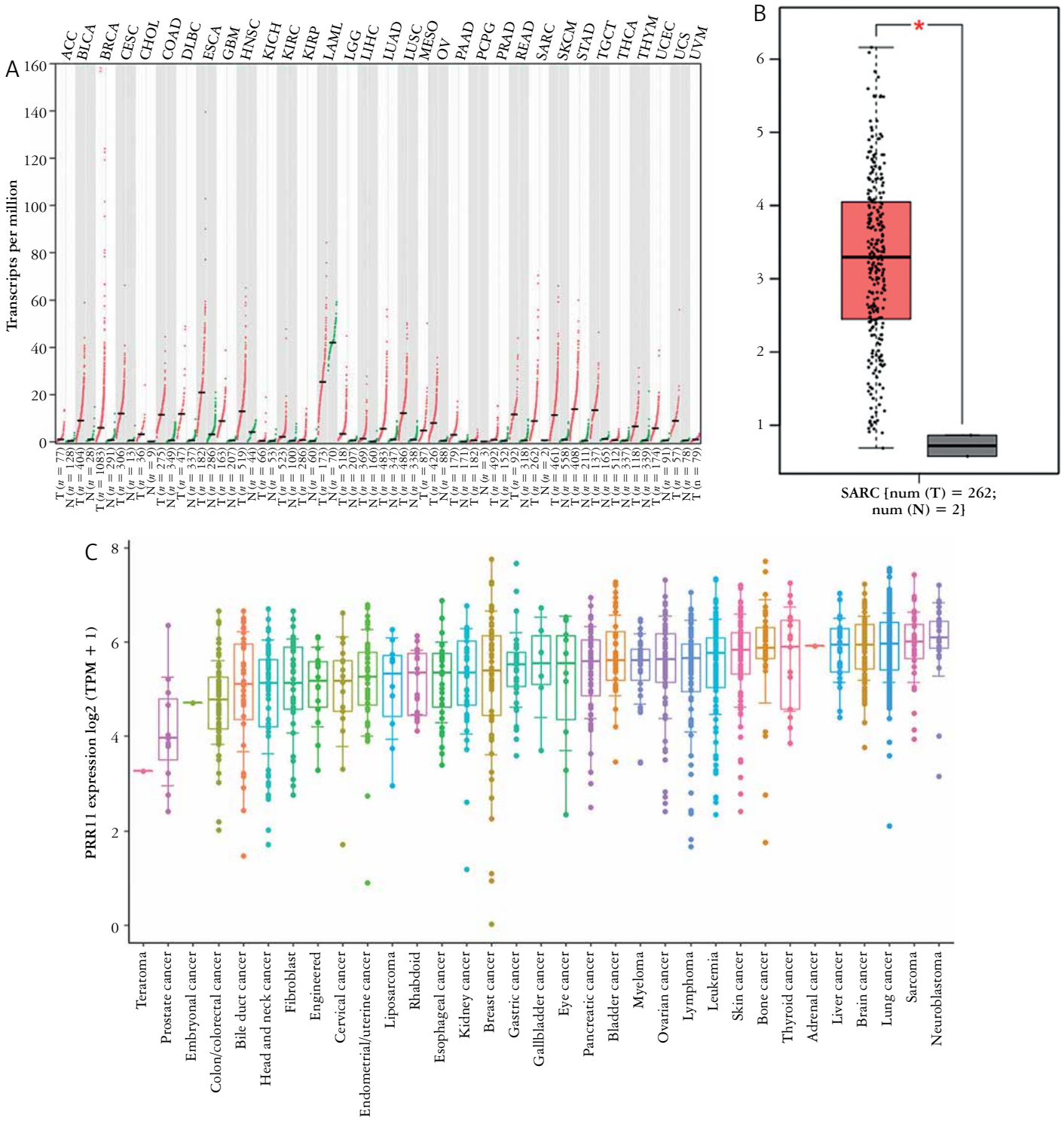

Fig. 1. PRR11 is expressed in malignant tumor cells. A) Expression levels of PRR11 mRNA in malignant tumors and normal tissues; B) expression level of PRR11 mRNA in sarcoma and normal tissues; C) PRR11 mRNA expression in malignant tumor cell lines

cell lines, with higher PRR11 expression in U2OS cells and lower expression in MG63 cells (Fig. 1D).

Survival analysis and clinical characteristics of PRR11 in osteosarcoma

The original data of patients with osteosarcoma were obtained from the TCGA database. After screening, there were 265 osteosarcoma samples with complete clinical information. The median expression of PRR11 was used to classify the samples into a high and a low expression group. The Kaplan-Meier survival curve showed that the overall survival and disease-specific survival of patients with osteosarcoma in the low PRR11 expression group were significantly

Table II. Effect of PRR11 and clinical characteristics on the prognosis in patients with osteosarcoma.

Clinical characteristics	Uni-variable Cox		
	HR	95% CI	*p*
PRR11	1.13	1.01–1026	0.035
Age	1.02	1.00–1.03	0.014
Gender	1.11	0.74–1.65	0.615
Pathologic tumor depth	1.04	1.02–1.06	0.000
Pathologic tumor length	1.06	1.03–1.08	0.000
Pathologic tumor width	1.02	1.01–1.03	0.00
Tumor multifocal	1.71	1.08–2.69	0.021
Tumor recurrence	2.34	1.39–3.96	0.001
Tumor metastasis	2.95	1.8–4.84	0.000
Radiotherapy	0.91	0.58–1.4	0.659
Tumor necrosis	1.2	0.95–1.52	0.136

higher than those of the high-expression group, and the difference was significant (Fig. 2A,B). However, there was no significant difference in the disease-free survival and progression-free survival in patients with osteosarcoma (Fig. 2C,D). To understand the independent prognostic indicators for survival in osteosarcoma, univariate regression analysis was performed on the clinical characteristics, and the results showed that PRR11 expression, age, tumor length, tumor depth, tumor width, tumor multifocality, tumor recurrence, and metastasis were the risk factors affecting the prognosis. However, radiotherapy, tumor necrosis percentage, and gender did not affect prognosis (Table II).

PRR11 gene set enrichment analysis pathway enrichment analysis in osteosarcoma

To study the enrichment pathways related to PRR11 in osteosarcoma, we used the gene set enrichment analysis (GSEA) software to perform pathway enrichment analysis on genes associated with PRR11 expression in osteosarcoma using the transcriptome data downloaded from the TCGA database. The results showed six pathways with significant significance ($p < 0.01$) (Table III). PRR11 is mainly involved in ubiquitin-mediated proteolysis, the cell cycle, and other pathways in osteosarcoma (Fig. 3).

Analysis of the protein interactions of PRR11

To explore the upstream and downstream relationships of PRR11 in osteosarcoma, the STRING was used, which showed that a total of 11 interaction nodes with $p = 2.53e{-}09$ were enriched. The genes with an interaction score > 0.400 were *CDC20*, *CCNA2*, *SKA2*, *GTSE1*, *TOP2A*, *CKAP2L*, *CKAP2*, *DLGAP5*, *AXIN2*, and *KIF11* (Fig. 4). These genes are mainly involved in cell cycle regulation, including sister chromosome segregation, and nuclear separation (Table IV).

Transfection of MG-63 cells with PRR11

The CCLE database and results of previous studies showed that the expression level of PRR11 was lower in MG-63 cells. Therefore, we selected the MG-63 cell line and transfected it with the high expression plasmid and the empty plasmid, respectively. Quantitative real-time polymerase chain reaction and Western blot showed that high-expression transfection effectively increased the level of PRR11 in MG-63 cells compared with the control group ($p < 0.05$, $^{**}p < 0.01$) (Fig. 5A,B).

PRR11 promotes proliferation of osteosarcoma MG-63 cells and inhibits apoptosis

Cell counting kit-8 assay revealed that after 24, 48, and 72 hours of transfection, the proliferation of the high expression group was significantly enhanced compared to the empty control group and blank control group (Fig. 6A). The flow cytometry revealed that compared to the empty group and the blank control group, the overall apoptosis rate of the PRR11 high expression group was reduced (Fig. 6B) ($p < 0.05$, $^{**}p < 0.01$).

PRR11 promotes invasion and migration of osteosarcoma cells

Next, we assessed the effect of PRR11 on migration and invasion of MG-63 cells. The results of the scratch test revealed that 24 hours after transfection, the migration rate of the high expression group was markedly increased compared to the empty control group and the blank control group (Fig. 7A). The results of the Transwell assay showed that 24 hours after transfection, cell invasion of MG-63 cells in the high expression group was increased compared to the empty control group and the blank control group (Fig. 7B) ($^{*}p < 0.05$, $^{**}p < 0.01$).

Discussion

Cell division is necessary for tissue growth and development, and mutations and deletions involving cell cycle regulation may lead to erroneous cell division and chromosome segregation, resulting in cell cycle disorders. Eventually, this may lead to serious diseases, such as tumorigenesis [13, 14]. There is increasing evidence that the inactivation of tumor suppressor genes or activation of proto-oncogenes can affect the cell cycle and lead to tumor initiation and progression [15, 16].

The tumor-related gene *PRR11* is a newly discovered gene situated on chromosome 17q22, consisting

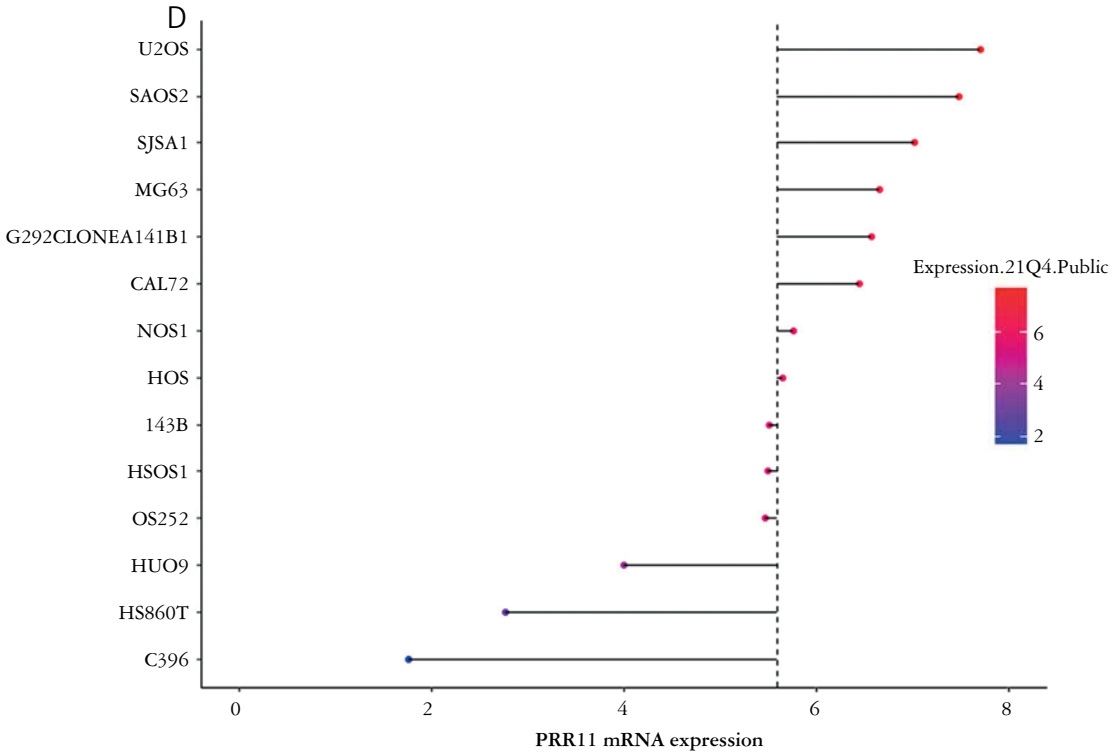

Fig. 1. Cont. D) expression levels of PRR11 mRNA in osteosarcoma cell lines

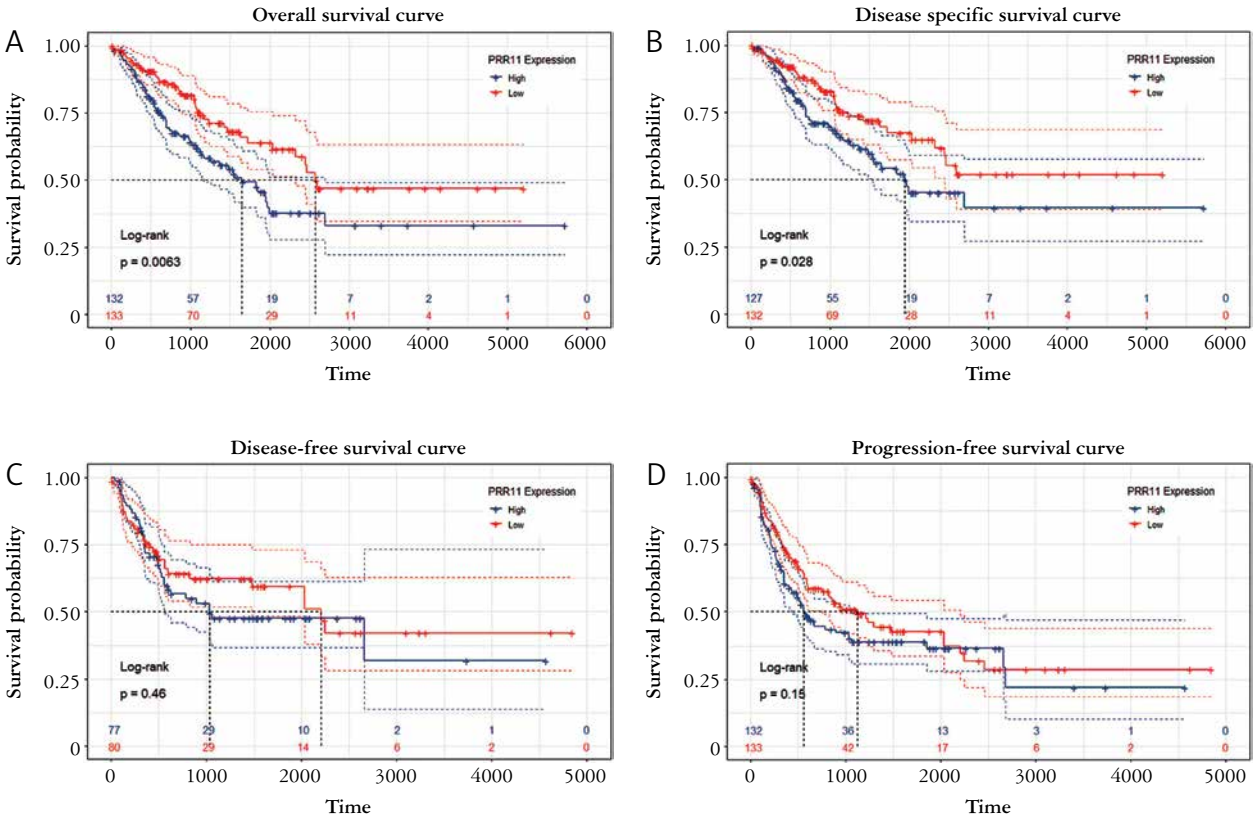

Fig. 2. Survival analysis of PRR11 in osteosarcoma. A) Overall survival in osteosarcoma; B) disease-specific survival in osteosarcoma; C) disease-free survival in osteosarcoma; D) progression-free survival in osteosarcoma

Table III. Gene set enrichment analysis of enriched pathways associated with PRR11 expression in osteosarcoma

KEGG signaling pathway name	Number of enriched genes	Enrichment score	Normalized enrichment score	*p*-value	FDR
KEGG_UBIQUITIN_MEDIATED_PROTEOLYSIS	130	0.35	2.15	0.000	0.010
KEGG_CELL_CYCLE	118	0.62	2.14	0.000	0.005
KEGG_OOCYTE_MEIOSIS	110	0.56	2.12	0.000	0.004
KEGG_BASAL_TRANSCRIPTION_FACTORS	35	0.59	1.92	0.004	0.033
KEGG_PROGESTERONE_MEDIATED_OOCYTE_MATURATION	85	0.52	1.91	0.002	0.027
KEGG_HOMOLOGOUS_RECOMBINATION	24	0.73	1.82	0.002	0.054

FDR – false discovery rate

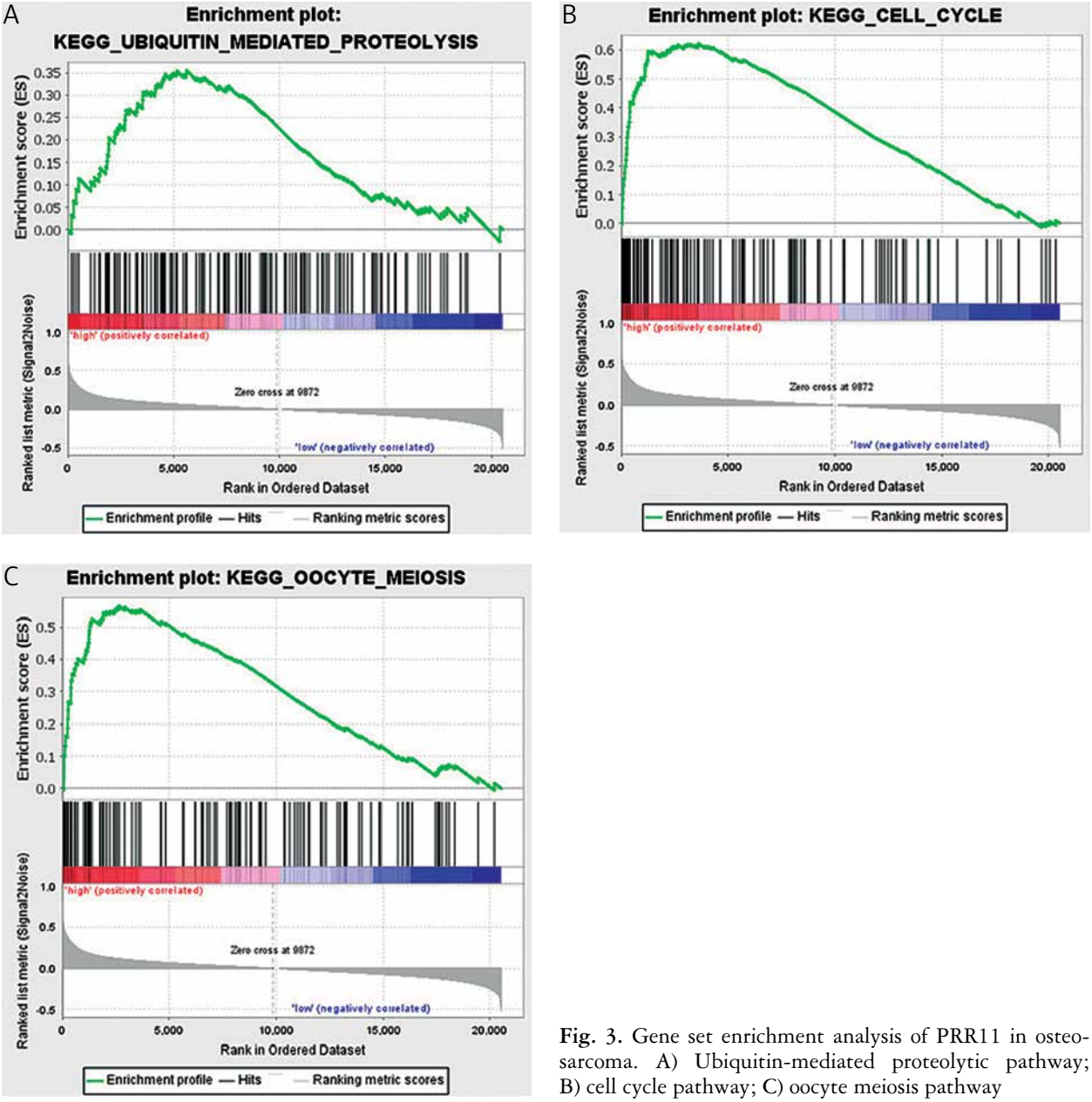

Fig. 3. Gene set enrichment analysis of PRR11 in osteosarcoma. A) Ubiquitin-mediated proteolytic pathway; B) cell cycle pathway; C) oocyte meiosis pathway

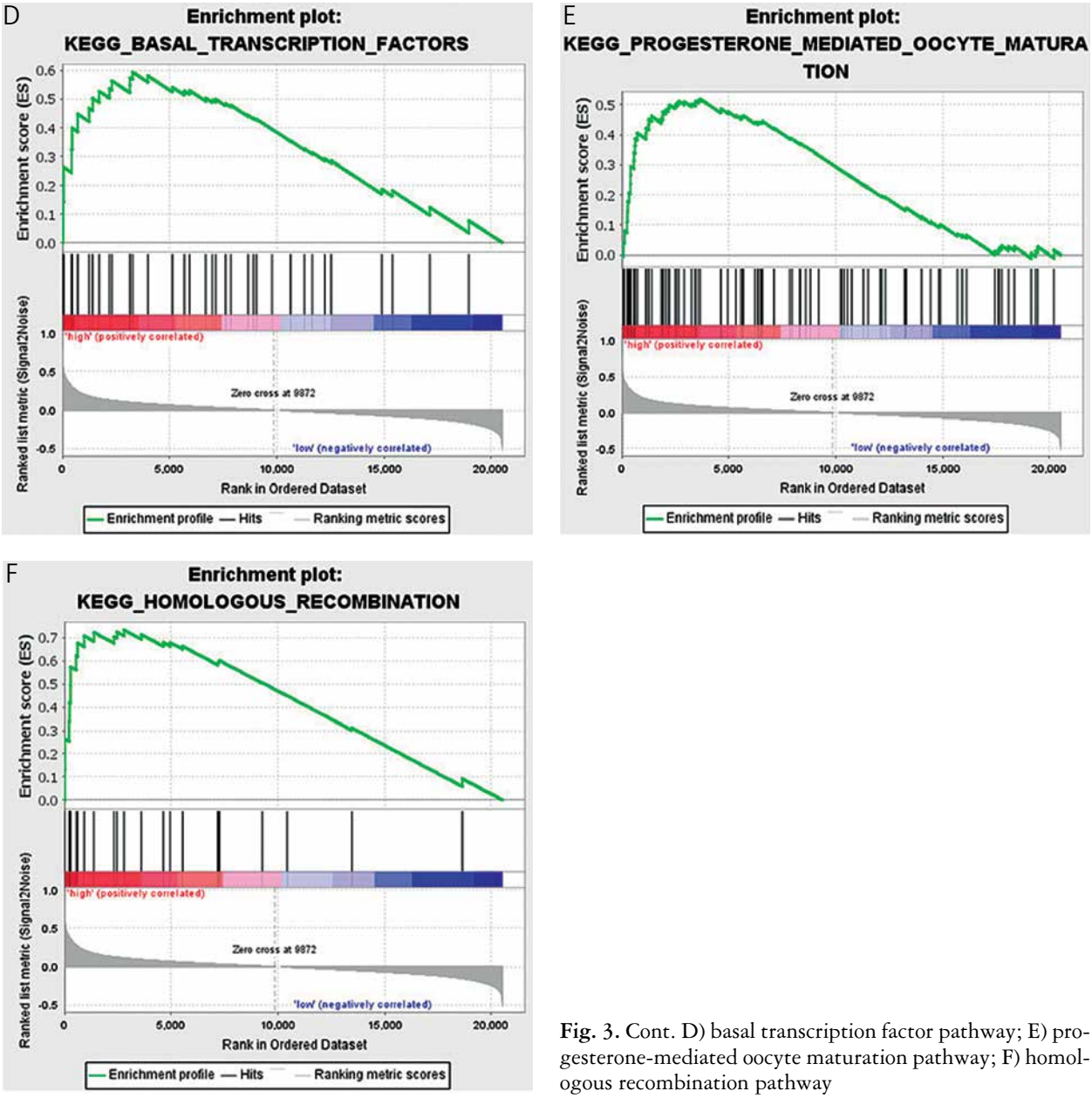

Fig. 3. Cont. D) basal transcription factor pathway; E) progesterone-mediated oocyte maturation pathway; F) homologous recombination pathway

of 10 exons and 9 introns, and the translation initiation codon is situated in the second exon, while the stop codon is situated in the last exon [17]. Many researchers have found that PRR11 is highly expressed in cancers such as colorectal, liver, breast, and ovarian cancer, and is closely related to poor prognosis [18–21]. However, only limited studies have reported the role of PRR11 in osteosarcoma. Song *et al*. found that miR-211-5p regulates PRR11 to promote apoptosis and inhibit the migration of osteosarcoma cells [12]. Our group previously found that the expression of PRR11 was elevated in osteosarcoma, and the proliferation, invasion, and migration of osteosarcoma cells were inhibited by downregulation of PRR11 [22]. In the present study, we used the GEPIA and CCLE databases and found that compared with normal tissues, the expression level of PRR11 is high in the vast majority of malignant tumors, including sarcomas. Also, the expression of PRR11 in osteosarcoma cells is high in a variety of malignant cell lines. The expression of PRR11 was the highest in U2OS cells, which was accordant with the finding of previous cell experiments.

Using bioinformatics methods, the relationship between genes and tumor prognosis can be analyzed quickly and cost-effectively. At the same time, data sets with multiple large sample sets are more reliable than single-sample studies. Hau *et al*. screened many differentially expressed pancreatic cancer genes including *PRR11* in TCGA, and the high expression of *PRR11* significantly shortened the five-year overall survival of pancreatic cancer and was an independent

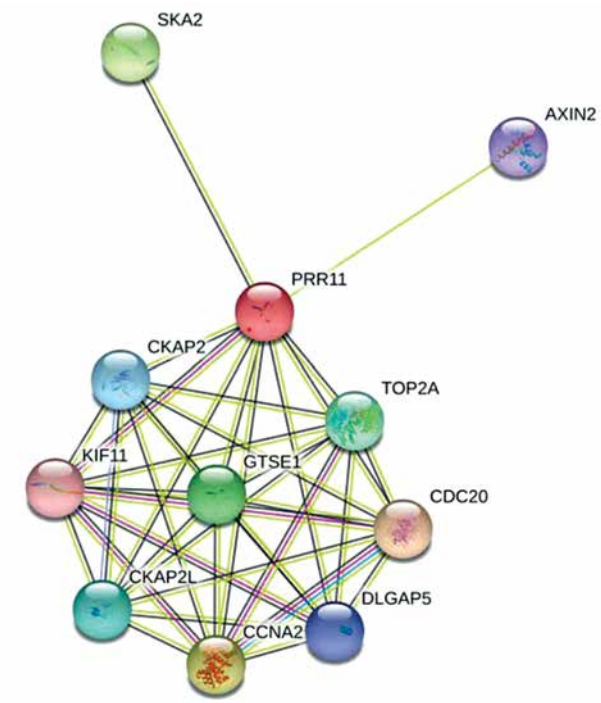

Fig. 4. Interacting protein analysis of PRR11 by protein-protein interaction network

risk factor [23]. Wang *et al*. found that the expression level of *PRR11* was related to the clinical stage, and T and N of TNM staging. Univariate and multivariate analysis showed that PRR11 was an independent prognostic factor for squamous cell carcinoma of the tongue [24]. In accordance with the TCGA database, the present study found that PRR11 had a significant effect on the survival rate of osteosarcoma, and the survival rate of the low expression group was significantly higher than that of the high expression group. In addition, in patients with osteosarcoma, age, tumor length, tumor depth, tumor width, tumor multifocality, tumor recurrence, and metastasis were the risk factors affecting prognosis. This is consistent with the above research results and provides evidence for the involvement of the *PRR11* gene in the progression of osteosarcoma.

Regarding the mechanism by which *PRR11* promotes tumor progression, many bioinformatics studies have shown that *PRR11* is mainly involved in regulation of the cell cycle, M phase, and mitotic cell cycle, and in various tumor signaling pathways [25–27]. In addition, a large number of cell experiments have also shown that *PRR11* affects cell migration, proliferation, and invasion in a variety of tumors. Zhou *et al*. found that silencing of *PRR11* inhibited the migration and proliferation of esophageal cancer cells and significantly up-regulated the expression of E-cadherin [28]. Wang *et al*. reported that overexpression of *PRR11* significantly increased the invasion and proliferation of tongue squamous cell carcinoma, while *PRR11* knockout had the opposite effect [29]. In the present study, through the GSEA pathway enrichment analysis, it was found that PRR11 was mainly enriched in pathways correlated with the cell cycle and cell division in osteosarcoma. Further PPI network analysis revealed that the proteins that bind to PRR11 are mainly CDC20, CCNA2, and SKA2, which predominantly participate in cell cycle regulation. We further verified the influence of *PRR11* on osteosarcoma cells through cell experiments. After the up-regulation

Table IV. Protein-protein interaction network of PRR11

Description	Count in network	Strength	FDR
Regulation of sister chromatid segregation	3 of 83	1.81	0.0188
Sister chromatid segregation	3 of 133	1.6	0.0417
Chromosome segregation	4 of 268	1.42	0.0179
Nuclear division	4 of 291	1.39	0.0197
Cell division	6 of 493	1.34	0.00024
Mitotic cell cycle	8 of 695	1.31	5.12e–06
Mitotic cell cycle process	7 of 616	1.31	3.03e–05
Regulation of chromosome organization	4 of 359	1.3	0.0313
Cell cycle process	8 of 976	1.16	2.45e–05
Regulation of mitotic cell cycle	5 of 676	1.12	0.0228
Cell cycle	9 of 1313	1.09	8.88e–06
Regulation of cell cycle process	5 of 760	1.07	0.313
Regulation of cell cycle	8 of 1230	1.06	8.94e–05
Regulation of organelle organization	7 of 1306	0.98	0.0029

FDR – false discovery rate

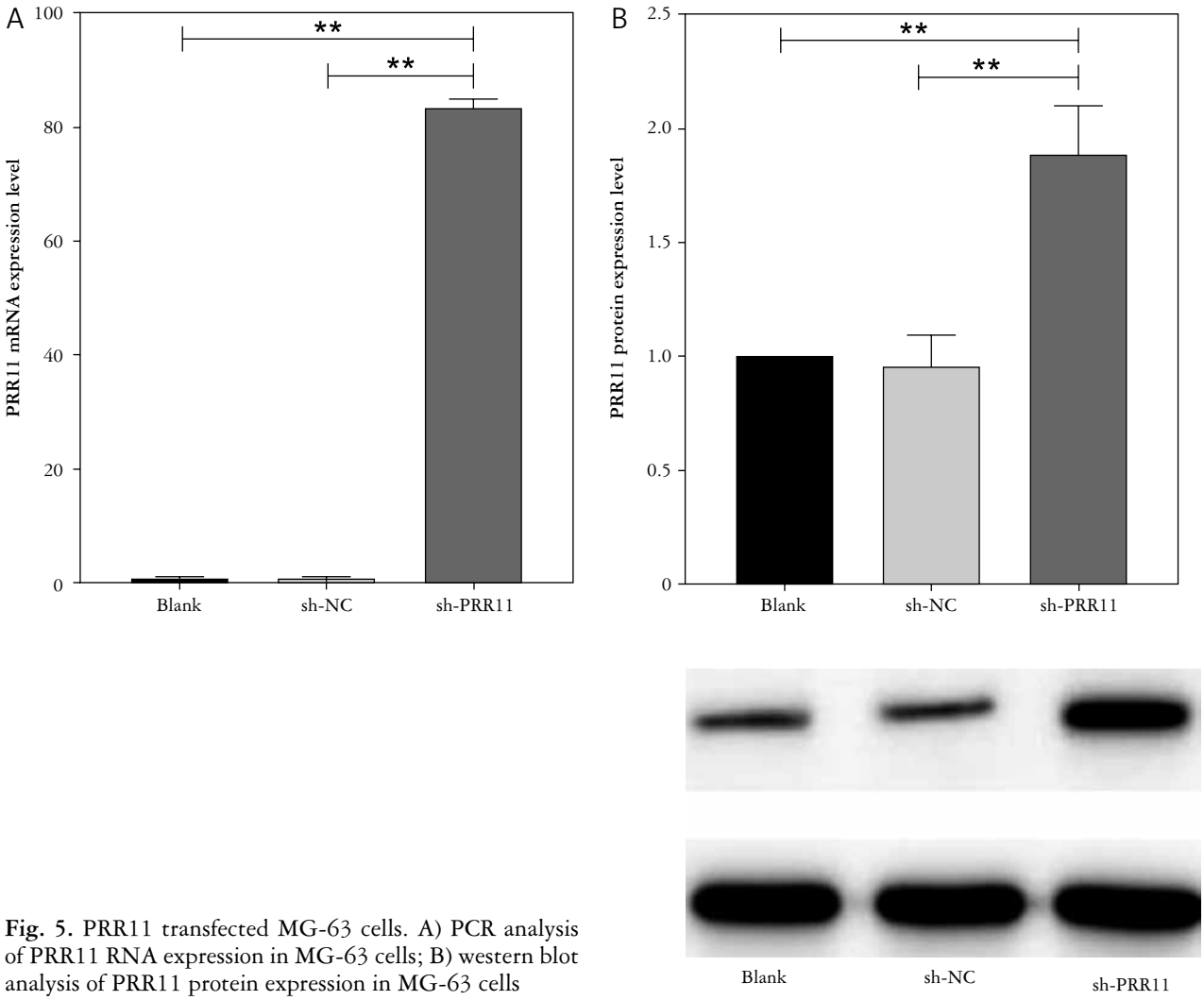

Fig. 5. PRR11 transfected MG-63 cells. A) PCR analysis of PRR11 RNA expression in MG-63 cells; B) western blot analysis of PRR11 protein expression in MG-63 cells

of *PRR11* in MG-63 cells, the migration, invasion, and proliferation abilities of osteosarcoma cells were enhanced, and apoptosis was weakened. This was consistent with the results of the above studies.

This study has some limitations. The molecular mechanism of *PRR11* regulating the cell cycle of osteosarcoma is still unclear. This research group will further study this at a later stage.

Conclusions

In summary, this study demonstrated the high expression of *PRR11* in a variety of malignant tumors *via* bioinformatics methods; moreover, the high expression is significantly correlated with prognosis. Gene set enrichment analysis and PPI network analysis revealed that *PRR11* is mainly involved in regulation of the cell cycle and other pathways in osteosarcoma. Up-regulation of *PRR11* promotes migration, invasion, and proliferation of osteosarcoma cells and inhibits apoptosis. These data strongly suggest that *PRR11* may be a predictor of survival in patients with osteosarcoma and a therapeutic target.

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The authors declare no conflicts of interest.

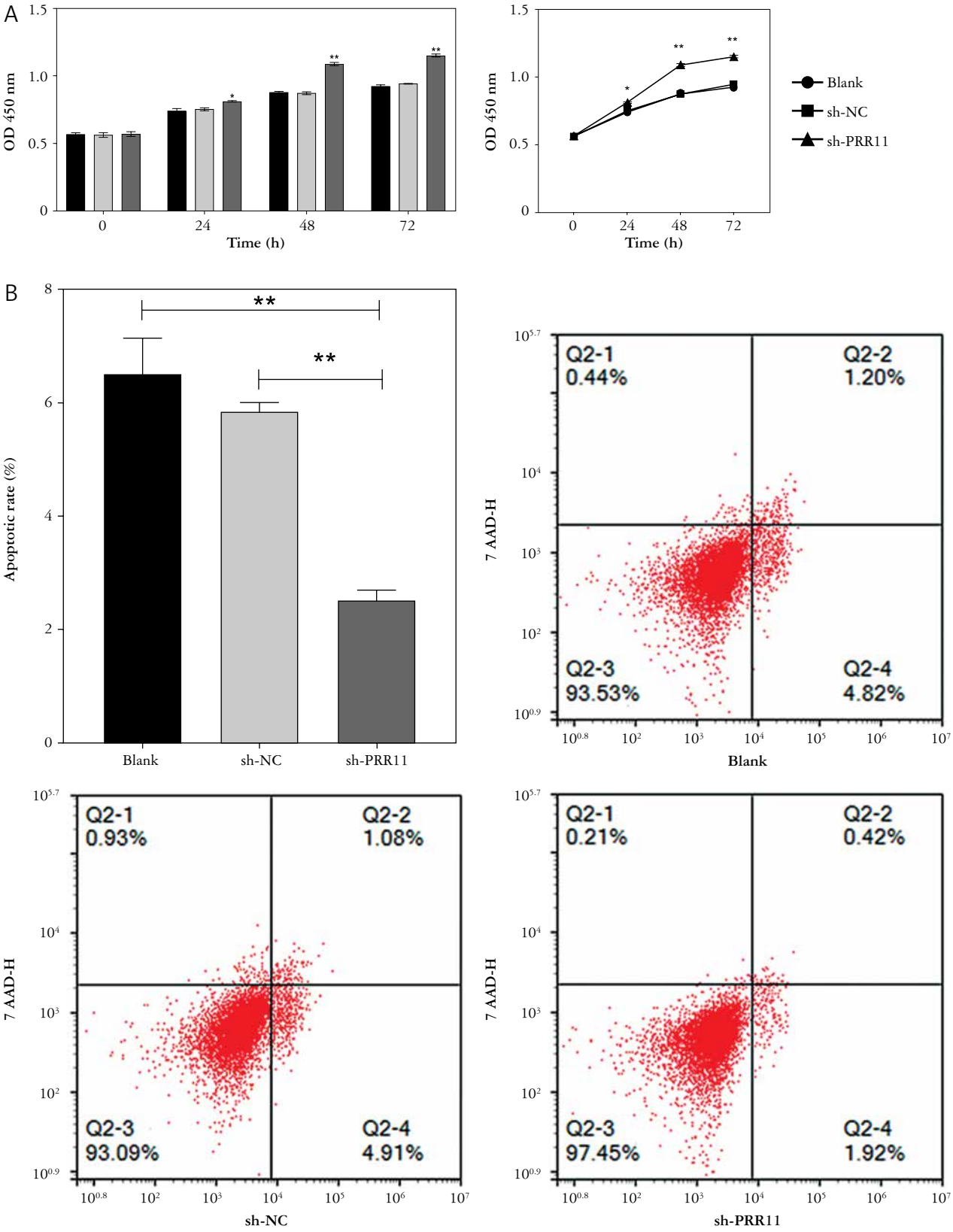

Fig. 6. PRR11 promotes proliferation of osteosarcoma MG-63 cells and inhibits apoptosis. A) Cell counting kit-8 assay for the proliferative ability of MG-63 cells after transfection at 24 hours, 48 hours, and 72 hours; B) the apoptosis level of transfected MG-63 cells was detected by flow cytometry

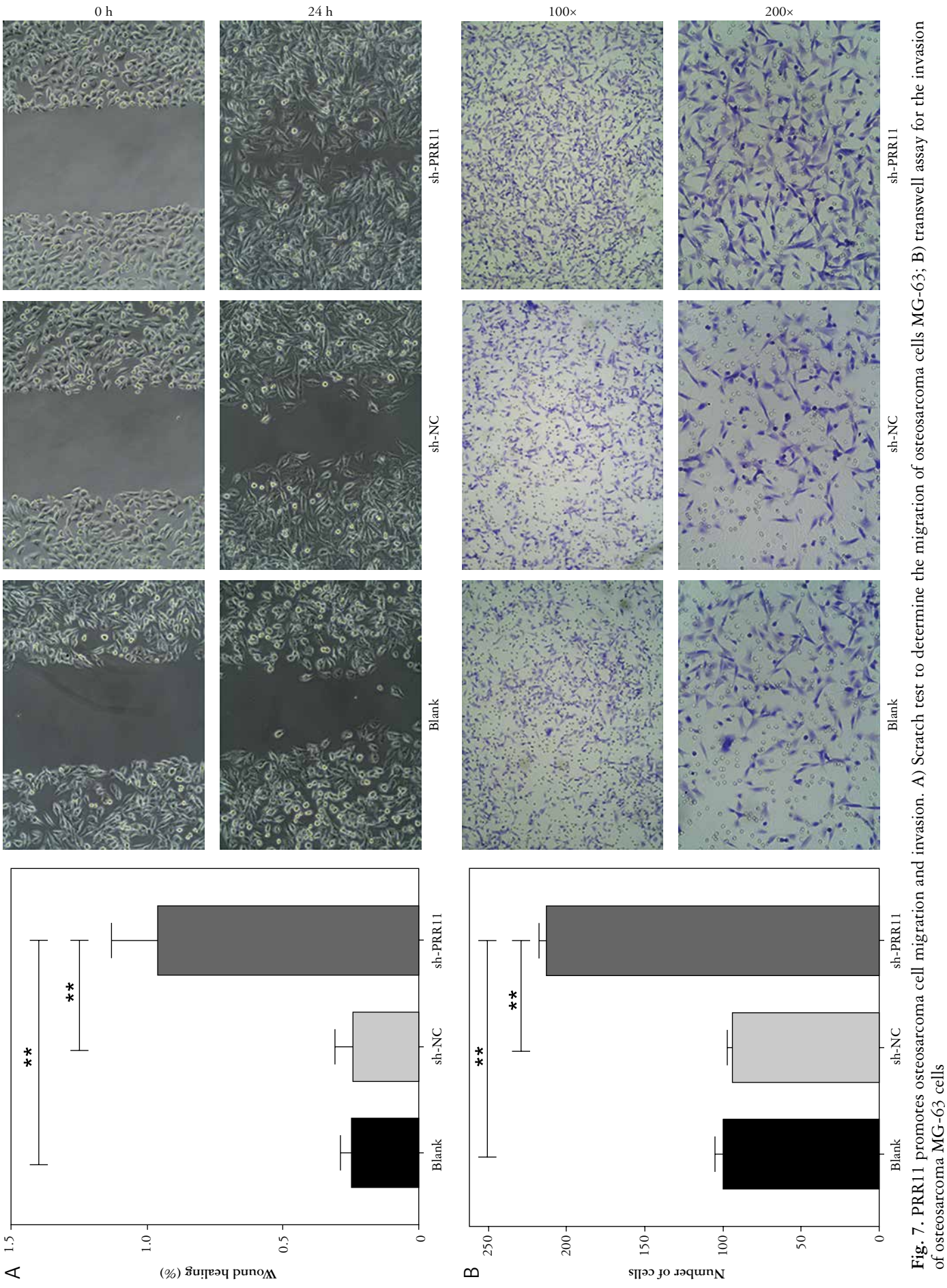

Fig. 7. PRR11 promotes osteosarcoma cell migration and invasion. A) Scratch test to determine the migration of osteosarcoma cells MG-63; B) transwell assay for the invasion of osteosarcoma MG-63 cells

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