Translational Oncology 15 (2022) 101253

Contents lists available at ScienceDirect

Translational Oncology

journal homepage: www.elsevier.com/locate/tranon

Original Research

SKA3 overexpression predicts poor outcomes in skin cutaneous melanoma patients

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ARTICLE INFO

Keywords:
SKA3
Skin cutaneous melanoma
Tumor microenvironment
Prognosis

ABSTRACT

Background: Spindle and Kinetochore Associated Complex Subunit 3 (SKA3) is a part of the SKA complex, which plays a key role in cell mitosis. Studies have shown that SKA3 was associated with cancer progression. However, its role in skin cutaneous melanoma (SKCM) remains unclear. Here, we investigated the expression level and prognostic value of SKA3 in SKCM.

Methods: Based on public databases, univariate and multivariate Cox regression analyses were used to investigate the different expression of SKA3 between SKCM and normal tissues. Then, the relationship between SKA3 expression level and prognosis was assessed. PPI network and functional enrichment analysis were performed. ESTIMATE and CIBERSORT were expected to evaluate the SKA3 expression and immune status. CCK8, wound healing, transwell assays and tumor xenograft trial were performed to detect the SKA3 function in cell viability, migration and invasion of the cell lines.

Results: The SKA3 was highly expressed in SKCM tissues. SKA3 overexpression was associated with poor survival and immune status. SKA3 knockdown inhibited cell viability, migration and invasion of SKCM cells.

Conclusion: SKA3 is involved in the progression of SKCM and may serve as a new prognostic biomarker and therapeutic target.

Introduction

Melanoma is the malignant tumor caused by uncontrolled proliferation of melanocytes and rank first among all skin cancer mortality rate [1,2]. Skin cutaneous melanoma (SKCM) is the most common melanoma, which triggered by ultraviolet radiation caused skin cell death and malignant transformation [3,4]. SKCM tends to metastasize to other tissues and organs, with a median survival rate of 8–12 months [5–7]. Early diagnosis and treatment will improve the prognosis. Therefore, the discovery of potential molecular markers that differently expressed in SKCM development may aid in the early diagnosis and treatment of the disease.

The SKA complex formed by three subunits, including SKA1, SKA2 and SKA3, is an essential component for accurate division of cells [8]. In mitosis, SKA3 is necessary for spindle checkpoint silencing and chromosome cohesion maintenance [9]. During metaphase, most of the chromosomes lined up at the equator of the cell due to activation of the spindle assembly checkpoint (SAC). At this point, if the supply of SKA3 exhausted, mitosis would arrest [10]. Studies have proved that SKA3 is dysregulated in many malignant tumors and closely related to prognosis. SKA3 overexpression would accelerate the adverse outcomes of breast cancer, lung adenocarcinoma and hepatocellular carcinoma [11–13]. However, the roles of SKA3 in the prognosis and development of SKCM have not been fully elucidated.

In solid tumors, the cancer cells are wrapped in an intricate mixture of nontumorous cells and matrix components which denominated as tumor microenvironment (TME). Accumulating evidence has indicated that tumor invasion and metastasis were the result of the co-evolution of cancer cells and microenvironment. Study have shown that SKCM is closely related to the immune microenvironment [14]. In the research, we verified the immune correlation between SKA3 and SKCM patients. With the invention of RNA sequencing technology and microarray technology, RNA research has become an indispensable part of medical research. Therefore, this study would also analyze the expression of SKA3 on the basis of multiple databases and explore the effect of SKA3 on SKCM *in vivo* and *vitro*.

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https://doi.org/10.1016/j.tranon.2021.101253
Received 30 July 2021; Received in revised form 12 October 2021; Accepted 20 October 2021

Materials & methods

Raw data

The mRNA expression of SKA3 in 472 SKCM patients was downloaded from The Cancer Genome Atlas (TCGA) database (https://genome-cancer.ucsc.edu/) [15]. We obtained 471 clinical data points for Cox analysis to evaluate the effect of SKA3 expression on the survival rate. Immune scores and stromal scores were generated by running ESTIMATE computational package [16].

Gene expression and Kaplan Meier survival analysis

Gene Expression Profiling Interactive Analysis (GEPIA) (http://gepia.cancer-pku.cn/) is an interactive website that compares upload data with Genotype Tissue Expression (GTEx) and TCGA data [17]. We used the GEPIA database to analyze the differences in SKA3 expression between normal and SKCM tissues as well as in different cancer stages. We also detected the relationship between SKA3 gene expression and overall survival (OS) and disease-free survival (DFS). Hazard ratios (HRs) and 95% confidence intervals (CIs) were used to assess relationships.

Gene set enrichment analysis

The search tool for the Retrieving of Interacting Genes/Proteins (STRING version 11.0; http://string-db.org) was applied to construct a protein-protein interaction (PPI) network of hub genes. A Minimum interaction score>0.4 was considered statistically significant [18]. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) analyses were performed on hub genes with the cluster Profiler, enrichplot and ggplot2 packages [19]. Gene set enrichment analysis (GSEA) was used to analyze the potential signaling pathways involved in the high vs. low expression of SKA3 in SKCM [20]. $P < 0.05$ and a false discovery rate (FDR)<25% were considered to indicate significant gene set enrichment.

Tumor-infiltrating immune cell profiles

CIBERSORT calculation method was used to estimate the distribution of Tumor-infiltrating immune cell (TIC) in all SKCM samples. The cases with $P < 0.05$ were selected for further analysis.

Prepare tissue samples and cell lines

The Human Protein Atlas (HPA) (http://www.proteinatlas.org/), a Swedish-based project in 2003, is dedicated to mapping all human proteins in cells, tissues and organs using a variety of omics technologies [21]. We compared SKA3 protein expression in skin and SKCM samples by assessing immunohistochemical images from the HPA. We also obtained 3 SKCM samples from Cancer Hospital of Jilin Province and 3 normal healthy skin samples from the Second Affiliated Hospital of Harbin Medical University (from 2020 to 2021). No patient had been treated before and all patients signed informed consent. All tissue samples were prepared for western blot. The suspended cell line SK-MEL-1 and the adherent cell line A-375 of SKCM were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HaCaT cells and human fibroblast cells (HFs) were donated by Dr. Chenyang Cui and Dr. Hao Wu.

Cell transfection

SK-MEL-1 and A-375 cell lines were transfected with short hairpin (sh)RNA to knockdown SKA3. Control and SKA3-specific shRNA were synthesized by Gene Pharma Co (Shanghai, China). Western blot to verified transfection efficiency.

Western blot assay

The soluble protein was prepared in RIPA lysis buffer (Beyotime, China) added with 1% phenylmethanesulfonyl fluoride (PMSF) (Beyotime, China). Equivalent protein (20–50 μg) was subjected to SDS-PAGE and transferred to electrophoretically PVDF membranes. After being immersed in protein free rapid blocking buffer (EpiZyme, China) at room temperature for 10 min, the membranes were incubated with specific primary rabbit antibodies antibody at 4 °C overnight, SKA3 (1:1000, SAB, USA), β-actin (1:5000, Abcam, UK), followed by incubated secondary antibodies (1:5000, Beyotime, China) at room temperature for 1 h. Then antigen-antibody complex was detected by enhanced chemiluminescence (ECL) reagent. β-actin were used as a loading control.

qRT-PCR analysis

According to the Trizol Reagent instructions, extract the total RNA of all tissue samples. Took 1 μl of RNA solution sample, used NanoDrop 2000 spectrophotometer to measure the absorbance value of A260/A280 and measured RNA concentration at the same time. Followed the instructions of ABScript II One Step SYBR Green RT-qPCR Kit (abclonal, China). The $2^{-\Delta\Delta Ct}$ method was used for analysis. The primer sequences of SKA3 and β-actin were listed in Table 1.

Cell viability assay

Cell count kit-8 (CCK8) assay (Beyotime, Shanghai, China) was utilized to detect cell viability. 1×10^4 cells were plated in 96-well plates and cultured in a complete medium (containing 10% Fetal Bovine Serum) at 37 °C/5% CO_2 overnight. Then measured the absorbance at 450 nm using a multiplate reader (BioTek, CA, USA) every 24 h.

Wound healing assay

We performed a wound healing test to observe if SKA3 has an effect on cell migration. Prepared the experiment when the cell density reaches 100% confluence. Used a 200 μl pipette tip on a single layer of cells to create a wound model and PBS to wash away the detached cells. The cells were cultured in a 37 °C/5% CO_2 incubator with serum-free medium. Took pictures with an inverted microscope at 0, 24, 48, and 72 h in order to measure the width of the scratch and evaluated the migration of cells.

Transwell migration and invasion assays

Put migration chambers in 24-well plates. With the presence or absence of Matrigel, add 100 μl serum-free medium in the chamber. Then placed 200 μl cell (2.5×10^5/ml in serum-free medium) in the chamber. Next, added 750 μl culture medium (with serum) in lower chamber. Incubated cells at 37 °C/5% CO_2 for 24 h. Removed the medium and washed twice with PBS. After fixing the cells by 4% formaldehyde at room temperature for 20 min, washed twice with PBS. Then crystal violet stain at room temperature for 20 min. Scrape off non-migrated cells with cotton swabs. Count migrated cells under a light microscope.

Tumor xenograft trial

We selected the SK-MEL-1 cell line to verify the effect of SKA3 on tumor formation ability. Healthy 4–6-week-old male BALB/c mice were randomly divided into control group and shSKA3 group. The back of the mice were selected for injection (100 μl), and the cell density was 1×10^8/ml. Observed the health status of the mice after the operation. Eat and feed normally. From 12 days after the operation, the tumor size was measured with a ruler. On 28 days, the mice were killed and the tumor

Table 1
Sequences of primers.

Gene	Forward primer	Reverse primer
SKA3	5′-CAGATCCCTCTTCACCTACGA-3′	5′-TCAACGTTTAAAGGGGGACA-3′
β- actin	5′-GGCATCCTCACCCTGAAGTA-3′	5′-GGGGTGTTGAAGGTCTCAAA-3′

was completely peeled off. The size and volume of the tumor were calculated. All procedures were approved by the Animal Care and Use Committee of the Second Affiliated Hospital of Harbin Medical University.

Statistics

x^2 test, Cox regression and all *in vitro/vivo* experiments data analysis were performed using SPSS version 19.0 (IBM Corp., Armonk, NY, USA) or GraphPad Prism 7.0 software, presented as mean ± SD. ROC curves were plotted using R. Comparison between groups was assessed using two-tailed paired or non-paired Student's *t*-test. *P*-values less than 0.05 ($P < 0.05$) was considered to indicate a significant difference.

Results

SKA3 overexpressed in SKCM

To investigate the potential prognostic implications of SKA3 in SKCM cases, we compared the expression level of SKA3 between normal and SKCM tissues by using the GEPIA dataset. The results showed that SKA3 was overexpressed in SKCM (Fig. 1A). Based on HPA database, the protein expression of SKA3 was analyzed. The immunohistochemical results indicated that no SKA3 expression was detected in normal skin tissues, while moderate expression was shown in SKCM slices (Fig. 1B). The same result was verified in western blot assay, the expression of SKA3 in SKCM was higher than in normal skin tissues (Fig. 1C). Furthermore, compared with HaCaT and HFs cells, SKA3 was highly expressed in SKCM cell lines (Fig. 1D). In addition, qRT-PCR analysis provided the same outcomes with the western blot (Fig. 1E, F). In summary, the above results confirmed that SKCM exhibited higher protein and transcriptional expression levels of SKA3 than normal tissue and cells.

Correlation of SKA3 expression with survival and clinicopathological factors in SKCM patients

Based on TCGA database, we assessed the association between SKA3 mRNA expression and clinicopathological characteristics of SKCM patients. We proved that SKA3 expression was related to Clark level, TNM stage and tumor recurrence (Fig. 2A). By using Kaplan Meier method, we further evaluated the relationship between the SKA3 expression and SKCM survival. The results indicated that SKA3 high expression was significantly associated with worse OS and DFS (Fig. 2B). Next, the association between clinicopathological factors and OS/DFS was determined by Cox logistic regression analysis. Univariate model analysis showed that OS was significantly associated with SKA3 mRNA expression, age, race, tumor size, lymph nodes and AJCC stage (Table 2), while DFS associated with SKA3 mRNA expression, age, gender, tumor size, lymph nodes, metastases, and AJCC stage (Table 3). Multivariate Cox model analyses further confirmed that high SKA3 mRNA expressions is an independent prognostic factor for both OS and DFS (Tables 2 and 3). The AUC values for the diagnosis of 1-year, 3-year and 5-year SKCM patients with SKA3 overexpression were 0.75, 077, and 0.75 (Fig. 2C). In conclusion, SKA3 had an independent prognostic effect on SKCM patients.

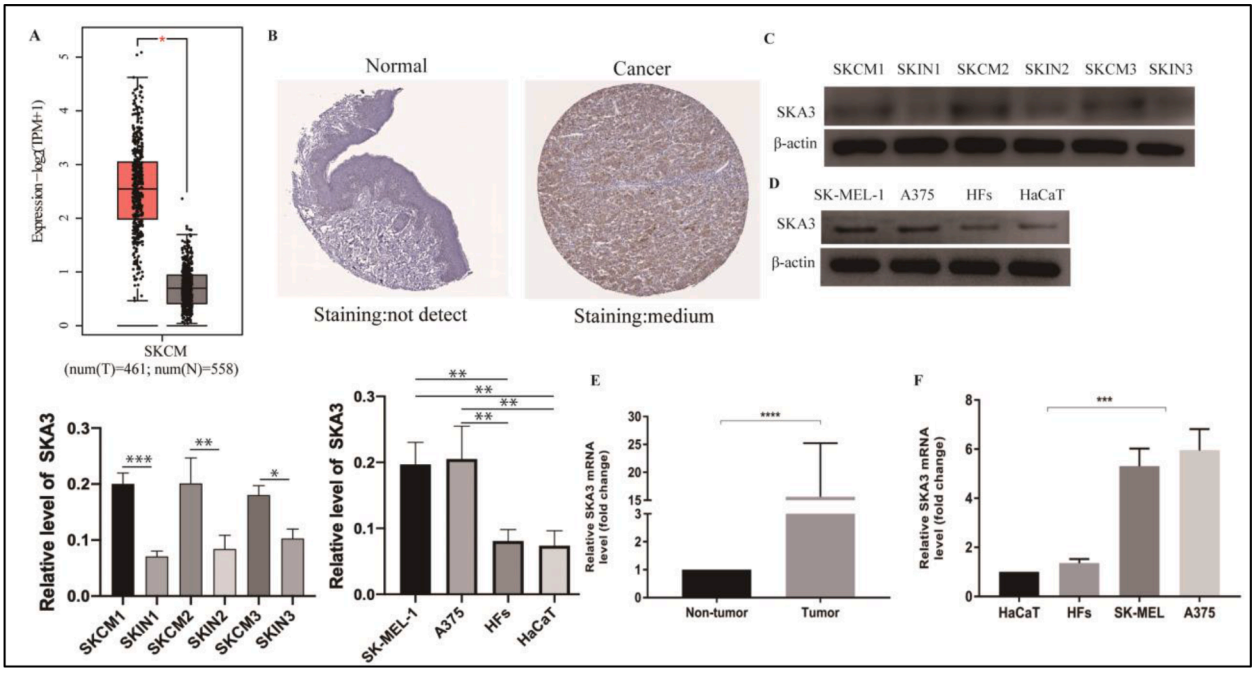

Fig. 1. The expression level of SKA3 in SKCM. A SKA3 expression between SKCM and normal tissues by using the GEPIA. B SKA3 in SKCM was analyzed using the HPA database. C Western blot studies showed the expression of SKA3 in SKCM. D Western blot assays showed the expression of SKA3 in SKCM cell lines. E mRNA expression of SKA3 in normal skin and SKCM. F mRNA expression of SKA3 in HaCaT, HFs and SKCM cells lines. Data were mean ± SEM. ($n = 3$). (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

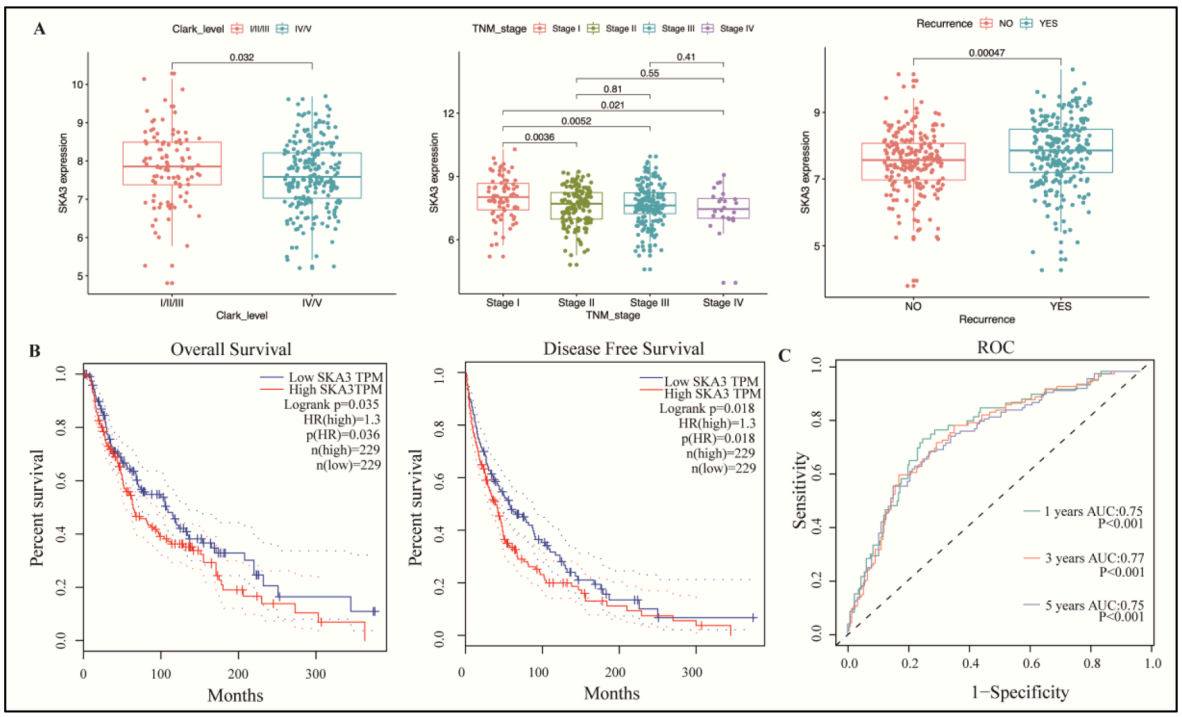

Fig. 2. SKA3 had independent prognostic effect on SKCM patients. A SKA3 expression with clinicopathological factors of SKCM patients. B SKA3 was associated with worse OS and DFS. C ROC curves was plotted to detect AUC values.

Table 2
Univariate and multivariate Cox logistic regression analysis of OS in TCGA cohorts.

Variates	Univariate analysis HR	95%CI	*P* value	Multivariate analysis HR	95%CI	*P* value
Age	1.734	1.312–2.292	0.000*	1.138	0.821–1.578	0.438
Gender	1.14	0.859–1.512	0.364	……	……	……
Race	0.351	0.172–0.715	0.004*	0.376	0.174–0.815	0.013*
pT	1.981	1.461–2.685	0.000*	2.198	1.565–3.086	0.000*
pN	1.767	1.316–2.372	0.000*	2.44	0.887–6.714	0.084
pM	1.716	0.906–3.251	0.098	……	……	……
AJCC Stage	1.626	1.216–2.173	0.001*	0.792	0.289–2.175	0.651
SKA3	1.044	1.044–1.002	0.039*	1.107	1.052–1.166	0.000*

Note: HR: hazard ratio; CI: confidence interval; AJCC stage: the extent of disease progression in cancer patients is described by the American Joint Committee on Cancer; TNM stage: tumor size, lymph nodes and metastases. (*$P < 0.05$).

Table 3
Univariate and multivariate Cox logistic regression analysis of DFS in TCGA cohorts.

Variates	Univariate analysis HR	95%CI	*P* value	Multivariate analysis HR	95%CI	*P* value
Age	1.769	1.274–2.457	0.001*	1.266	0.866–1.851	0.223
Gender	1.404	1.0–1.97	0.05*	1.313	0.897–1.92	0.161
Race	0.509	0.188–1.379	0.184	……	……	……
pT	1.927	1.355–2.742	0.000*	2.299	1.535–3.442	0.000*
pN	2.139	1.523–3.004	0.000*	2.865	1.011–8.122	0.048*
pM	2.004	1.019–3.945	0.044*	2.485	1.036–5.957	0.041*
AJCC Stage	2.106	1.499–2.958	0.000*	0.866	0.3–2.504	0.791
SKA3	1.063	1.015–1.113	0.009*	1.131	1.069–1.196	0.000*

Note: HR: hazard ratio; CI: confidence interval; AJCC stage: the extent of disease progression in cancer patients is described by the American Joint Committee on Cancer; TNM stage: tumor size, lymph nodes and metastases. (* $P < 0.05$).

PPI network and functional enrichment analysis of SKA3

By STRING network construction, ten genes significantly coregulated with SKA3 were identified, including SKA1, SKA2, BUB1B, CCNB1, BOD1, SPDL1, BUB1, NDC80, PLK1 and CENPF (Fig. 3A). To clarify the biological role of these 10 synergistic regulatory proteins, functional enrichment analysis was performed (Fig. 3B). To further assess the potential role of SKA3 in SKCM, GSEA was conducted on high and low expression groups, respectively. The top functions and pathways were plotted. Above results indicated that in GO, KEGG and HALLMARK enrichment analyses, high expression of SKA3 was positively correlated with cell cycle, DNA replication. Low SKA3 expression group mainly enriched in glycosaminoglycan degradation, autophagosome maturation and P53 pathway (Fig. 4A–F). In the C7 gene set collection defined by MSigDB, both SKA3 high and low expression groups were enriched in numerous immunologic and immune functional gene sets (Fig. 4G, H).

In addition, we also screened the differentially expressed genes related to SKA3 by comparing RNA-sequencing (RNA-seq) data of low vs. high SKA3 expression cohorts and identified 16 up-regulated genes and 18 down-regulated genes (fold change>1.5, $P < 0.05$). By GO and KEGG enrichment analysis the most significant features are concentrated in miRNA metabolic process and miRNAs in cancer (Fig. 5A, B). Therefore, the overall function of these genes seems to be related to cell division, protein regulation and cancer regulation.

Overexpression of SKA3 was associated with the immune status of SKCM

Recent studies have confirmed that the TME played an important role in the occurrence and development of SKCM, and ESTIMATE algorithm could be used to evaluate prognosis. By calculating the immune and stromal scores, we divided all SKCM samples into low and high score groups respectively, and the analysis confirmed that patients with high SKA3 expression had lower scores (Fig. 6A). To further explore the potential association between SKA3 and immune infiltration in the SKCM TME, we implied CIBERSORT computational method. The difference analysis showed that SKA3 expression negatively correlated with T cells

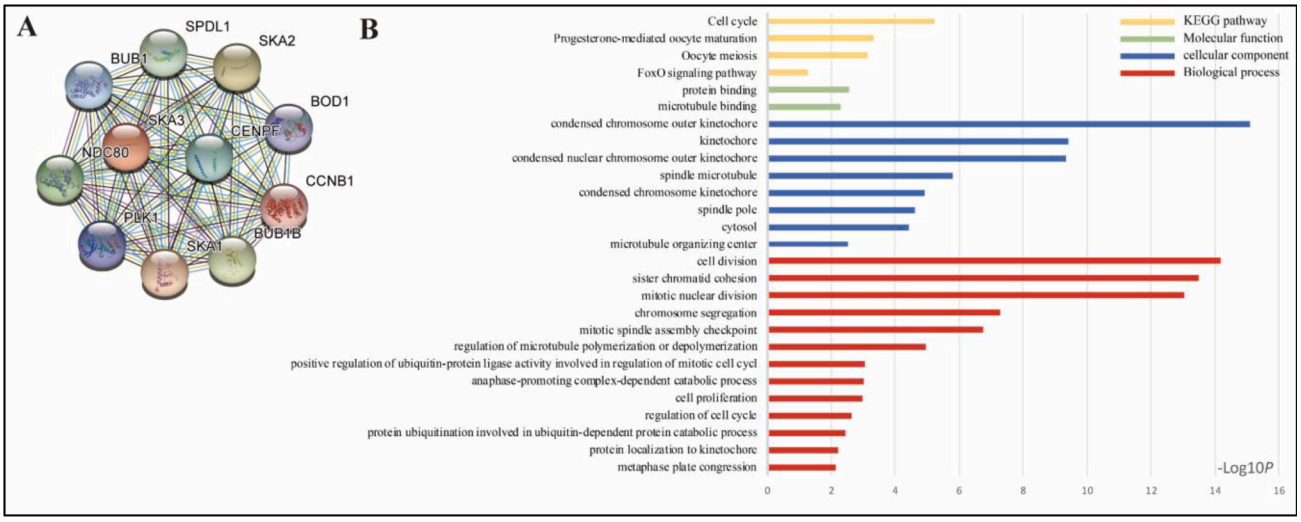

Fig. 3. PPI network and functional enrichment analysis of SKA3. A coregulated genes with SKA3. B Functional enrichment analysis to clarify the biological effect.

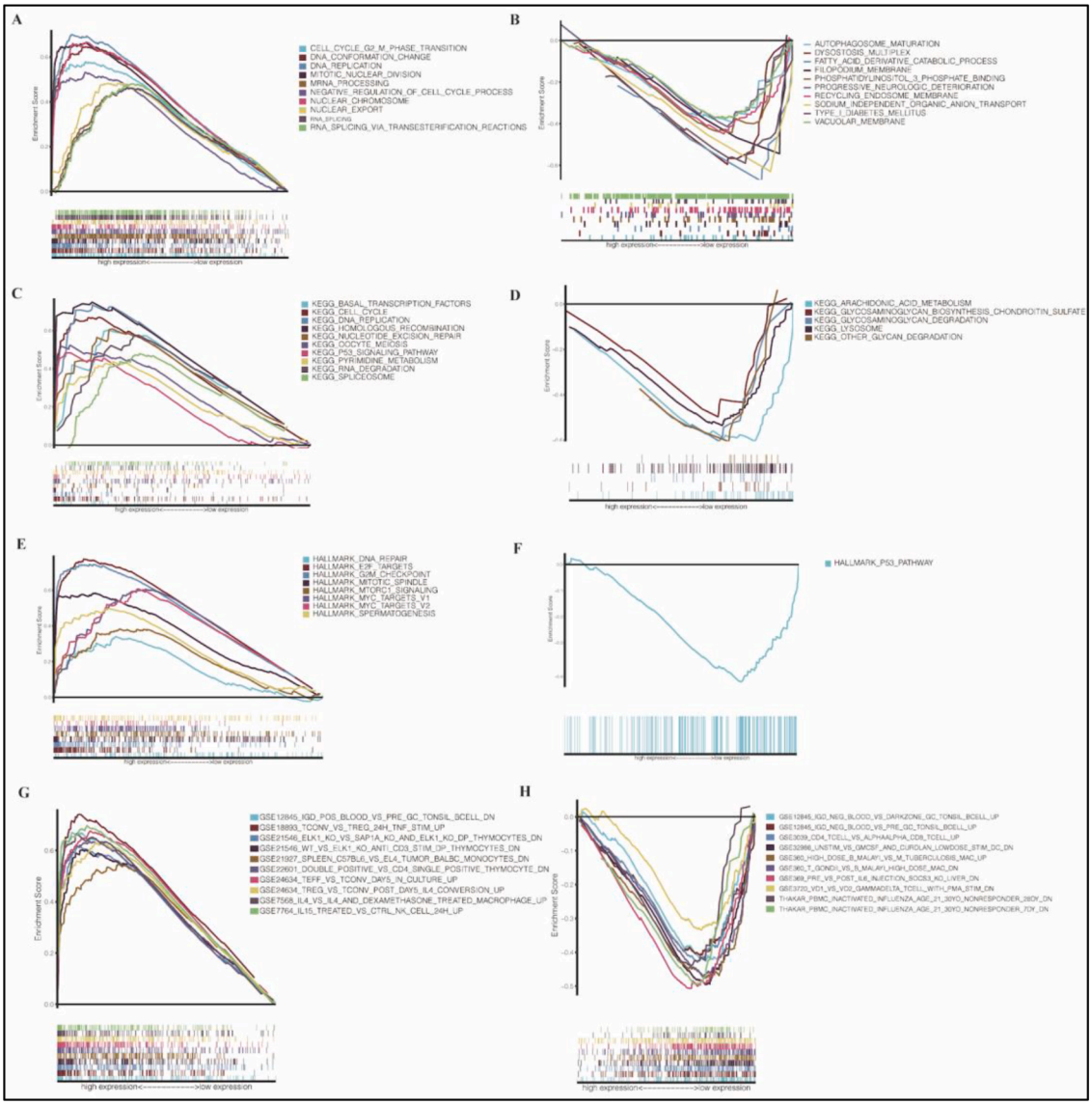

Fig. 4. Used GSEA to estimate the potential role of SKA3 in SKCM. A, B GO enrichment analyses was performed. C, D KEGG pathway analysis. E, F HALLMARK was assessed. G, H C7 enrichment analyses was conducted.

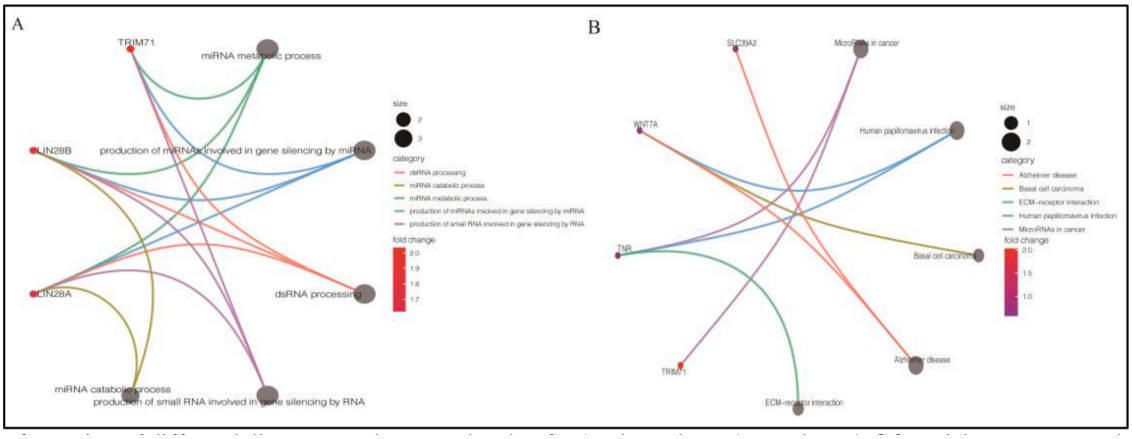

Fig. 5. Screening of differentially expressed genes related to SKA3 through RNA-seq data. A GO enrichment was conducted. B KEGG enrichment was performed.

regulatory (Tregs) and positively correlated with Macrophages M1 (Fig. 6B). In correlation analysis, the infiltration of Macrophages M1, Macrophages M2, T cells follicular helper, Tregs and B cells memory were related to SKA3 expression (Fig. 6C). These results further demonstrated that SKA3 expression would influence the immune activity of SKCM TME.

SKA3 knockdown inhibited the cell viability, migration and invasion of SKCM cells

To verify the role of SKA3 in the SKCM, we reduced the expression of SKA3 in SK-MEL-1 and A375 cell lines. Western blot test was used to verify the transfection efficiency (Fig. 7A). To investigate whether SKA3 could effect the cell viability of SKCM, we conducted the CCK8 assays in SK-MEL-shSKA3, SK-MEL-shCtrl, A375-shSKA3 and A375-shCtrl cells. The results showed that declined the expression of SKA3 could obtain higher cell viability level (Fig. 7B). Function acquisition assays demonstrated that low SKA3 expression could recede the migration and invasion of A375 cells (Fig. 7C, D). *In vivo* experiments, the same volume of SK-MEL-1-shSKA3 and SK-MEL-1-shCtrl cells were injected subcutaneously into BALB/c nude mice. The tumor appeared on the 12th day after the inoculation, and the metastasis in the SK-MEL-1-shSKA3 group was significantly suppressed compared with the control group on the 28th day after the injection (Fig. 7E).

Discussion

Melanoma accounts for only a small fraction (3%) of all skin cancers diagnosed each year, but is responsible for the vast majority (65%) of deaths [22]. Compared with most cancers, the incidence of SKCM varies greatly between different races. It is well-known that ultraviolet radiation would induce malignant transformation or death of skin cells and be considered as the primary risk factor for melanoma [23]. The increased melanin barrier in darker-pigmented individuals decreases both ultraviolet (UV) A and B radiation that pass through the skin [24]. However, SKCM rates are higher in Caucasian populations and this change is partly attributable to lower melanin content, which leads to reduced photoprotection [25]. Despite huge progress has been made with the innovation of diagnosis and treatment technology, SKCM is still a major clinical challenge. At present, the most effective treatment for melanoma is surgical resection, however for some inoperable advanced patients even chemotherapy and targeted therapies are generally less effective [26]. Therefore, it is an urgent issue to detect potential molecular targets and prognostic biomarkers for the diagnosis and treatment of SKCM.

Tumor occurrence is closely related to abnormal cell division. In mitosis, cell divides into two identical daughter cells by generating bipolar spindle. The precise chromosomes separation in this process depends on the firmly connection between chromosomes and the tips of dynamic microtubules [27]. SKA complex is essential for stable motion-microtubule binding, and SKA3 is the key to microtubule binding regulating. In addition, SKA3 directly interacts with tubulin monocytes or participates in SKA3-SKA1 microtubule interaction, which has been confirmed to be associated with the occurrence and development of certain tumors [27,28]. In the manuscript, for the first time we confirmed that SKA3 was overexpressed in SKCM and demonstrated the association with clinicopathological features as well as prognosis. The results suggested that SKA3 overexpression was related to SKCM recurrence, Clark level, TNM stage and poor prognosis. SKA3 knockdown inhibited the viability, migration and invasion of tumor cells both *in vitro* and *vivo*. The above consequences indicated that SKA3 overexpression accelerated tumor growth and might serve as a predictor of SKCM poor prognosis. In addition, from the analysis results, we speculated that SKA3 high expression in the early stages of cancer development was caused by abnormal cell proliferation. As for advanced stages, tumor burden increased and proliferation slowed down, in this period, if SKA3 expression remained unchanged, indicated that tumor cells had a strong ability to replicate, which might lead to tumor recurrence and high mortality.

Furthermore, we screened SKA3 related hub genes including CCNB1 and PLK1. Previous research has confirmed that SKA complex could regulate CCNB1 degradation and promote the mitosis transition from metaphase to anaphase [29]. CCNB1 downregulated would induce cell cycle arrest and even block the melanoma lung metastasis [30]. In NRAS mutant melanoma cells, the expression of mitotic regulator PLK1 increased, and the combined treatment of MEK inhibitor and PLK1 inhibitor could induce apoptosis and synergistically decline the proliferation of NRAS mutant melanoma [31].

Moreover, melanoma is one of the most immunogenic tumors and immunotherapy should have a good therapeutic effect. However, the tumor has acquired a mechanism to evade immune system detection, which leading to immune escape [32,33]. TME is crucial to many aspects of tumorigenesis, including tumor angiogenesis and metastasis. It has been proved to influence the response to antitumor therapies. Hence, lucubrating tumor stromal may improve the curative effect of current treatment methods and provide new opportunities for therapeutic targeting. For instance, cancer-stroma interaction would rain influence on the efficacy of cytotoxic regents and boosting number of neutrophils induced by given granulocyte (G)-CSF might lead to tumor aggressiveness increasing [34–36]. In the manuscript, through further analysis, we could conclude that overexpression of SKA3 was associated with the immune scores and stromal scores of SKCM and may play a role in SKCM

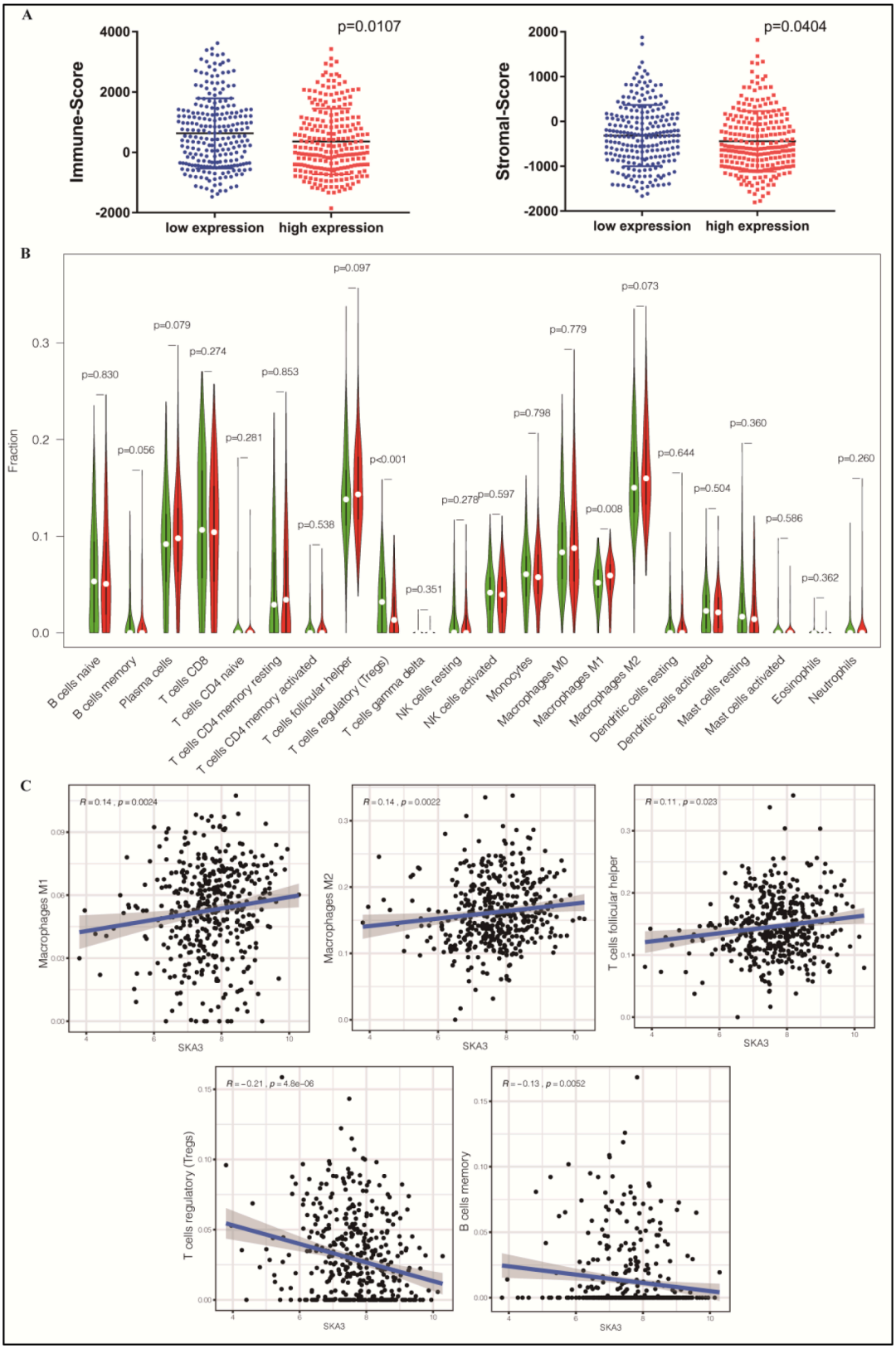

Fig. 6. Overexpression of SKA3 was associated with the immune status of SKCM. A The immune and stromal scores were analyzed. B Difference analysis showed that SKA3 expression correlated with the level of immune infiltration. C Correlation analysis showed that SKA3 expression correlated with the level of immune infiltration.

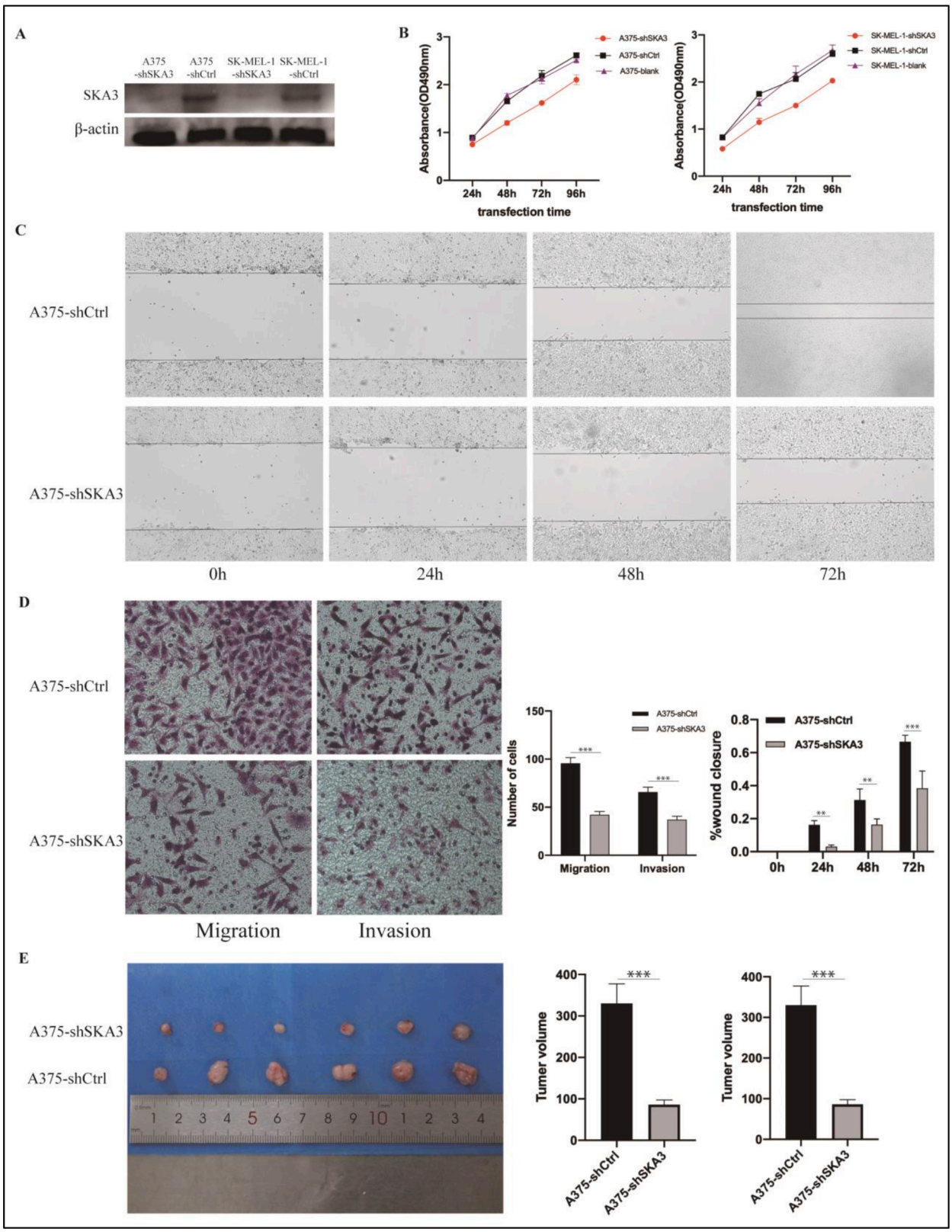

Fig. 7. SKA3 overexpression could affect tumor biological processes. A Lentivirus transfection efficiency. B CCK8 detected the cell viability. C Wound healing was performed with SKA3 knockdown. D Transwell assays was detected with low expression of SKA3. E Tumor formation ability was verified by Tumor xenograft trial. Data were mean ± SEM. ($n = 3$) (**$P < 0.01$; ***$P < 0.001$).

TME. We had also proved that SKA3 expression was significantly correlated with the infiltration level of immune cells, including Macrophages M1, Macrophages M2, T cells follicular helper, Tregs and B cells memory. Beside, certain specific cell microenvironment is a critical factor for spindle orientation regulation, which would determine the initial fate of daughter cells [37,38]. Whether the TME would regulate SKCM cell division through SKA3 needs further experimental research.

Conclusion

In summary, SKA3 was overexpressed in SKCM, downregulating whose expression could attenuate the viability, migration and invasion of tumor cells. SKA3 also associated with the immune status of SKCM and could possibly be used as an indicator of TME state transition. Hence, further research should be proceeded to clarify the molecular mechanism of SKA3. Targeting SKA3 is expected to be a new treatment strategy for SKCM.

CRediT authorship contribution statement

Hao Pang: Writing – original draft, Data curation, Writing – review & editing. **Yongting Zhou:** Investigation, Writing – review & editing. **Jie Wang:** Investigation, Writing – review & editing. **Hao Wu:** Investigation, Writing – review & editing. **Chenyang Cui:** Investigation, Writing – review & editing. **Zhibo Xiao:** Investigation, Writing – original draft, Data curation, Writing – review & editing.

Declaration of Competing Interest

The authors declared that they have no competing interests.

Acknowledgments

Not applicable.

Funding

This work was supported by the National Natural Science of China (Grant 81471796) and Heilongjiang Province Science Foundation for Excellent Youth Scholars (Grant JC2017019).

Ethical approval

This study was conducted by the Institutional Review Committee of the Second Affiliated Hospital of Harbin Medical University and was conducted in accordance with the Helsinki Declaration 2013.

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