

N2-neutrophils Promote Invasion and Metastasis of Ovarian Cancer by Upregulating MAPK Signaling

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Research

Keywords: N2-neutrophils, MAPK signaling, Ovarian cancer, invasion and metastasis

Posted Date: August 19th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-59568/v1>

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Abstract

Background: Metastasis is an important factor of high mortality in ovarian cancer. Neutrophils are involved in multiple pathologic mechanisms of cancer, including invasion and metastasis. However, the relationship of neutrophils and invasion and metastasis in ovarian cancer is unclear, as well as the exact mechanism.

Methods: To verify the relationship of neutrophils and invasion and metastasis in ovarian cancer, we tested the expression of CD11b in 24 groups of benign and malignant ovarian tumor tissues. And then, we tested the expression of CD11b, CXCL8, and CXCR1 in 16 cases of ovarian cancer, including primary lesions, metastatic lesions and adjacent carcinoma tissues. We successfully build tumor associated neutrophils research model (N1 and N2) and prove that N2-neutrophils can promote the invasion and metastasis of ovarian cancer. Next, we screened the significantly changed MAPK signaling pathway by high-throughput sequencing. And then confirmed this conclusion by molecular biology experiments.

Results: The expression of CD11b was significantly higher in malignant tumor than benign tumor tissues tested by western blot and Immunohistochemistry. The expression of CD11b, CXCL8 and CXCR1 is highest in ovarian cancer

metastases, followed by the primary lesions, and then the adjacent carcinoma tissues tested by PCR and WB methods. We proved that N2-neutrophils can promote the invasion and metastasis of ovarian cancer by transwell assay. Furthermore, we detected the related indicators of metastasis including MMP-2, MMP-9, E-Cadherin, N-cadherin and Vimentin by PCR and WB methods. Next, we screened the significantly changed MAPK signaling pathway by High-throughput sequencing through comparing ovarian cancer cells before and after co-cultured with N2-neutrophils. At last, we found the key gene P38 of MAPK signaling pathway by molecular biology experiments.

Conclusions: N2-neutrophils promote the invasion and metastasis of ovarian cancer by Upregulating MAPK signaling pathway, find a key gene P38.

Introduction

Ovarian cancer is one of the common malignant tumors in the female reproductive system, and the mortality rate ranks first among all kinds of gynecological tumors. More than 20,000 women worldwide are diagnosed with ovarian cancer each year¹. In the past 30 years, the clinical outcome of ovarian cancer has not improved significantly, and the overall 5-year survival rate of patients is 25% to 30%². Ovarian cancer lacks typical clinical features and specific diagnostic indicators in the early stage. It is often asymptomatic before metastasis. Most patients are already in advanced stage (FIGO III-IV stage) when diagnosed. Even accepted timely surgery and chemotherapy, only a small percentage of patients have achieved some relief, and most patients in advanced stage will relapse within 18 months. Many ovarian cancer patients experienced chemotherapy resistance after receiving multiple chemotherapy, which can not control local, distant metastasis effectively, leading to death³.

In condition of acute and suppurative infections, various poisoning, tissue damage, malignant tumors, etc., the number of neutrophils will increase significantly⁴. CD11b positive cells are granulocytes, mainly neutrophils, followed by macrophages. In mouse tissues, CD11b+ Ly6G+ cells are neutrophils, while CD11b+ Ly6G- cells are macrophages⁵. CXCL8, also known as IL-8, is a small molecule peptide secreted by macrophages, T cells, neutrophils and epithelial cells. CXCR1 and CXCR2 are co-ligands of CXCL8 and are mainly expressed on the surface of neutrophils⁶.

Neutrophils are highly mobile, and are rapidly recruited to the tumor microenvironment in the early stages of the tumor. We refer to neutrophils infiltrating in the tumor microenvironment as tumor-associated neutrophils (TANs). As early as 2009, Fridlender et al. demonstrated the existence of two different forms of tumor-associated neutrophils⁵. In the early stage of tumorigenesis, neutrophils chemotaxis to malignant sites, inhibiting the occurrence and development of tumors through cytotoxicity and immune activation, we defined this group as N1- neutrophils. In the middle and late stages of the tumor, neutrophils accumulate in the tumor stroma. The phenotypic and functional characters changed under the action of factors produced by tumors and microenvironment cells. This type of cells can promote tumor cell proliferation, migration, invasion and angiogenesis, and inhibit the body's anti-tumor immunity, which was defined as N2- neutrophils⁷. The plasticity of TAN is regulated by various cytokines in the tumor microenvironment. Studies have shown that N2- neutrophils can promote the development of tumor cells, participate in angiogenesis, and enhance the invasion and metastasis of tumor cells by secreting various cytokines and chemokines, biological agents such as NE, MMP-9 and ROS, presenting a tumor-promoting effect⁸⁻¹⁰.

TANs has been confirmed to be associated with the development and prognosis of various malignant tumors in humans, such as kidney cancer, liver cancer, gastric cancer, pancreatic cancer, colon cancer, malignant melanoma, cholangiocarcinoma, and uterine cancer¹¹⁻¹⁶. However, there are few studies on neutrophils and ovarian cancer. The main focus of research in the past five years on neutrophil-lymphocyte ratio (NLR) can be used to assess the prognosis of patients with ovarian cancer¹⁷⁻¹⁹. The relationship between neutrophils and invasion and metastasis of ovarian cancer has rarely been reported^{20,21}, and the exact molecular mechanism is still unclear. This article intends to confirm the relationship between neutrophils and invasion and metastasis of ovarian cancer. Furtherly, we strive to seek for its possible molecular mechanism to explore new potential therapeutic targets for ovarian cancer.

Materials And Methods

Patients and specimens

All clinical pictures and tissue specimens were taken from the sample library of the Tenth People's Hospital affiliated to Tongji University (2012-2016). The patients were enrolled with informed consent, and the specimens were collected and approved by the hospital ethics committee. All patients were

confirmed by pathology. No treatment measures such as radiotherapy and chemotherapy were received before surgery, and other systemic neoplastic diseases were excluded at the same time.

Immunohistochemistry

The Immunohistochemical staining was performed on paraffin-embedded tissues according to the manufacturer's instructions of EnVision kit (MaiXin Biotech Co., Fuzhou, China). The primary antibody was used rabbit anti-human CD11b monoclonal antibody (1:100, Abcam, Cambridge, UK ab133357). The immunohistochemical scoring principle was according to the staining intensity (no signal=0, weak=1, moderate=2, high=3), and the percentage of staining cells (0=0%; 1= 1%-25%; 2= 26%-50%; 3= 51%-75%, 4= 76%-100%). The final score of 0-12 was based on multiplying the scores of intensity and percentage. The staining scores of CD11b ≥ 4 was considered as high expression, <4 being regarded as low expression.

Cell culture and plasmid transfection

SKOV3 cells purchased from ATCC and cultivated in McCoy's

medium (Biochrom AG, Berlin, Germany) containing 10% fetal bovine serum at 37°C in a 5% CO₂ humidified atmosphere. OVCAR3 cells purchased from ATCC (Manassas, Virginia, USA) were cultivated in RPMI medium (Invitrogen) containing 10% fetal bovine serum. HL60 cells purchased from were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and cultivated in RPMI medium (Invitrogen) containing 10% fetal bovine serum which was induced by 1.25% DMSO. TGF- β -RNAi lentiviral vectors were purchased from Shanghai GeneChem Company (Shanghai, China). and the shRNA control sequence was 5'-GGACTATCCACCTGCAAGA-3'.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol (Invitrogen, 15596-018). First strand cDNA synthesis was performed using a reverse transcription kit (TransGen Biotech, Beijing, China) and the real-time PCR analyses were conducted on an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) using the SYBR Green dye (Roche Diagnostics, Mannheim, Germany). The GAPDH gene was used as an internal control, and the data are shown as the fold change. The experiment was performed in triplicate. Primers for MMP-2, MMP-9, E-Cadherin, N-cadherin, Vimentin and GAPDH are shown in Table 1.

Table 1
Primers for PCR

Gene	Forward sequences	Reverse sequences
MMP-2	CCCACTGCGGTTTTCTCGAAT	CAAAGGGGTATCCATCGCCAT
MMP-9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC
E-Cadherin	CGAGAGCTACACGTTACCGG	GGGTGTCGAGGGAAAAATAGG
N-cadherin	TCAGGCGTCTGTAGAGGCTT	ATGCACATCCTTCGATAAGACTG
Vimentin	AGTCCACTGAGTACCGGAGAC	CATTTACGCATCTGGCGTTC
GAPDH	GGAGCGAGATCCCTCCAAAAT	GGCTGTTGTCATACTTCTCATGG

Western blotting

Total protein was extracted in RIPA lysate with PMSF 1mM (Solarbio, Co. Ltd, Beijing, China), and quantified with BCA method. A total of 30 μ g of protein was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDSPAGE), followed by transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The PVDF membranes were incubated with primary antibody: anti-CD11b antibody (1:1000, Abcam, Cambridge, UK, ab133357), CXCR4 (1:1000, Abcam, ab124824), CXCR1 (1:1000, Abcam, ab14936), CXCL8 (1:1000, Abcam, ab18672), CCL5 (1:1000, Abcam, ab108933

), TGF- β (1:1000, Abcam, ab235178), MMP2 (1:1000, Abcam, ab92536), MMP9 (1:1000, Abcam, ab38898), Vimentin (1:1000, Abcam, ab92547), E-cadherin (1:1000, CST, 3195T), N-cadherin (1:1000, CST, 13116T), p-Erk (1:1000, CST, 4370s), Erk (1:1000, CST, 4695s), p-P38 (1:1000, CST, 4631s), P38 (1:1000, CST, 8690s), p-JNK (1:1000, CST, 4668s), JNK (1:1000, CST, 9252S), GAPDH (1:1000, CST, 5174) at 4°C overnight. After the membranes were incubated with horseradish peroxidase (HRP) conjugated secondary antibody Anti-mouse IgG (HRP) (1:5000, CST, 7076) and Anti-rabbit IgG (HRP) (1:5000, CST, 7074) and visualized by chemiluminescence ECL detection system (Bio-Rad).

Transwell assay

Transwell assay was used to evaluate cell migration and invasion. For migration assay, 4×10^4 ovarian cancer cells as control groups (SKOV3 and OVCAR3) were seeded in the upper in serum-free culture medium (200 μ l), Ovarian cancer cells 4×10^4 and N2 neutrophils 2×10^4 co-culture as experimental group. The lower chamber filled with complete medium. The cells were fixed with 4% paraformaldehyde and stained with gemsa 15min, after 24h incubation. The images were acquired under microscope and

migrated cells were counted in 5 random fields. The method of invasion assay was similar to migration, but the upper chamber coated with matrigel (BD Bioscience, Bedford, MA, USA).

High-throughput sequencing

Total mRNA of OVCAR3 and OVCAR3-N2 were extracted from cell by Trizol reagent (Invitrogen) separately. The RNA quality was checked by Bioanalyzer 2200 (Aligent) and kept at -80°C. The RNA with RIN >8.0 is right for experiment. The

complementary DNA (cDNA) libraries were prepared using the NEBNext™ Ultra Directional RNA Library Prep Kit, Next PolyA

mRNA Magnetic Isolation Module. Next Multiplex Oligos according to the manufacturer's instructions. The products were purified and enriched by PCR to create the final cDNA libraries and quantified by Agilent2200. The tagged cDNA libraries were pooled in equal ratio and used for 150 bp paired-end sequencing in a single lane of the Illumina HiSeqXTen. HTseq was used to count gene and RPKM method was used to determine the gene expression. We applied DESeq2 algorithm to filter the differentially expressed genes.

Statistical analysis

Statistical analysis was performed using GraphPad Prism Version 5.0 and SPSS 19.0 were performed to analyze data which were presented as value ± standard deviation. Comparisons between 2 groups were performed by standard Student's t test. $p < 0.05$ was considered to indicate a significant difference (* $p < 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Results

Neutrophils infiltration in ovarian cancer

It is observed that ovarian cancer metastasis is common in the peritoneum, intestine, mesentery, and omentum surface. Late ovarian cancer often shows ascites, local necrosis of cancer, and similar purulent infection (Figure 1A-D)

It is well known that there is a large amount of neutrophils in the infection area when inflammation occurs. We examined the expression of neutrophil marker CD11b in 24 pairs of ovarian benign (serosal cystadenoma) and malignant tumors (serous cystadenocarcinoma) by western blot and immunohistochemistry. The western blot showed that the expression of CD11b in ovarian malignant tumor tissues was significantly higher than that in ovarian benign tumor tissues (Figure 2A).

Immunohistochemical examination revealed that there were few neutrophils in benign ovarian tumor tissues; neutrophils were distributed in malignant tumor tissues, and the proportion of positive cells was more than 50%. Through statistical analysis, it is suggested that the condition of neutrophils infiltration were significant different between the two groups. ($p \leq 0.001$) (Figure 2B). These results demonstrate that neutrophil infiltration is closely related to ovarian cancer.

We selected 16 pairs of ovarian serous carcinoma samples (including primary lesions, metastatic lesions, and adjacent tissues), and detected mRNA and protein expression of neutrophil CD11b, CXCL8 and CXCR1 by PCR and WB methods. The results showed that these indicators were the highest in ovarian cancer metastasis tissues, followed by primary tumor tissues, and the expression of adjacent tissues was the lowest (Figure 2C). This result suggests that neutrophils may have a relationship with ovarian cancer metastasis.

N2 -neutrophils promote invasion and metastasis of ovarian cancer cells

We then applied the *in vitro* experiment to confirm the above human clinical data. Using HL60 cells as the initial cells, a TGF- β stable knockout cell line was constructed (HL60-si TGF- β). HL60-si TGF- β cells and HL60 cells were induced with 1.25% DMSO for 5 days to construct TAN cell models named N1- neutrophil and N2- neutrophil separately. Cell models was verified by western blot (Figure 3A). First, We selected two ovarian cancer cell lines SKOV3 and OVCAR3, co-cultured with N2- neutrophils for 48 hours, and then examined the changes of cell invasion and metastasis ability in the control and treatment groups. The results showed that the invasive and metastatic ability of ovarian cancer cells was significantly enhanced after co-culture with N2- neutrophils (Figure 3B-C). Next, We selected a well-cultured ovarian cancer cell line OVCAR3 and OVCAR3-N2 (OVCAR3 co-cultured with N2-neutrophils for 48 hours) to extract mRNA and protein, and detected the expression of metastasis-related indicators. Results showed that the expression level of these indicators significantly changed after co-cultured with N2-neutrophils (Figure 3D-E). The above data prove that N2-neutrophils can promote the invasion and metastasis of ovarian cancer cells.

Mechanism of N2-neutrophils promoting the invasion and metastasis of ovarian cancer

In order to clarify the molecular mechanism by which N2-neutrophils promote invasion and metastasis of ovarian cancer, we performed high-throughput sequencing. The well-cultured cell line OVCAR3 (control group) and OVCAR3-N2 (treatment group) were selected to extract mRNA, and then perform high-throughput sequencing to analyse differentially expressed genes. Heatmap results are showed (Figure 4A). Path analysis of the differentially expressed genes detected the significantly changed MAPK signaling pathway. The results are as follows (Figure 4B).

MAPK is an important transmitter of signal transduction from the cell surface to the interior of the nucleus. MAPK subfamilies include ERK, p38, JNK and so on. We selected two ovarian cancer cell lines [SKOV3 and OVCAR3], co-cultured with N2- neutrophils for 48 hours, and then examined the changes of key factors of MAPK signaling pathway in the control and treatment groups by western blot method and p-P38 was detected (Figure 5A). SB-202190 is a highly selective, potent and cell-permeable inhibitor of p38 MAP kinase. We added SB-202190 to the experimental system and then detect the expression of p-P38 by western blot methods. The results showed that p-P38 expression was significantly increased after co-culture with N2-neutrophils compared with the mono-inhibitor group (Figure 5B). The above data confirmed that N2- neutrophils can promote ovarian cancer invasion and metastasis by upregulating the phosphorylation level of P38, a key factor in MAPK signaling pathway.

Discussion

In recent years, the mechanism of action of neutrophils in tumor metastasis has emerged in an endless stream. Neutrophils can promote tumor cell metastasis by degrading extracellular matrix and promoting tumor cell clonal formation²². Increases the invasive ability of bladder cancer cells by regulating the regulatory signaling pathways of androgen receptor (AR)/MMP13 signaling pathways²³. Promotes invasion and metastasis of renal cancer cells by activating VEGF α / HIF2 α and estrogen receptor β signaling²⁴. Coffelt et al. demonstrated that ($\gamma\delta$) T cell-derived IL-17 can promote breast cancer tumor cell metastasis by inducing neutrophils to activate-CD8+ T lymphocyte activity²⁵. Neutrophils can significantly increase the potential of ovarian cancer cells to invade and migrate by inducing EMT transformation in tumor cells²⁰. The MAPK signaling pathway can promote the invasion and metastasis of tumor cells by degrading the extracellular matrix and affecting the adhesion of tumor cells²⁶. We analyzed differentially expressed MAPK signaling pathways by high-throughput sequencing. We found that the expression of p-P38 was significantly increased in tumor cells after co-culture with neutrophils. So it needs to be further studied how neutrophils affect the phosphorylation level of P38 and how p-P38 affects the invasion and metastasis ability of tumor cells. Szczerba et al. from the University of Basel recently published an article entitled neutrophils escort circulating tumour cells to enable cell cycle progression in Nature, which reported that neutrophils interact with partially circulating tumor cells to promote cancer cell cycle progression and metastasis²⁷. How neutrophils interact with ovarian cancer cells to promote tumor progression is also a problem we are very concerned about and is the focus of our next step.

The important role of neutrophils in tumors suggests that targeting neutrophils may have potential anti-tumor value. Since tumor and microenvironment-derived factors can induce local aggregation of neutrophils to tumors and promote malignant progression of tumors, the use of specific antibodies to antagonize neutrophil chemotaxis can play an anti-tumor effect. C-X-C chemokine receptor 1/2 (CXCR1/2) is an important receptor mediating neutrophil chemotaxis, Using CXCR1/2 neutralizing antibodies and small molecule inhibitors can reduce neutrophils infiltration and tumor growth. In addition, the induction of neutrophil transformation from tumor-promoting to tumor-suppressing may also be a new tumor treatment strategy. So far, mechanisms for regulating neutrophils to inhibit tumorigenesis or

promote tumor growth have not been fully understood. The phenotypic and functional heterogeneity of neutrophils in tumors has not been fully elucidated. Assessing the prognosis of tumor patients by testing neutrophils infiltration, as well as the activation of neutrophil anti-tumor effects in the treatment of cancer still need more experimental and clinical research evidence support, especially in ovarian cancer. With the deepening of research, neutrophils and related factors are expected to become new targets for molecular diagnosis and treatment of ovarian cancer.

Conclusion

The expression of neutrophils in ovarian malignant tumors was significantly higher than that of ovarian benign tumor tissues tested by WB and immunohistochemistry. In vitro experiments show that the ability of ovarian cancer cells to invade and metastasize is significantly enhanced after co-culture with N2 neutrophils. Further, we analyzed the MAPK signaling pathway with significant changes through high sequencing analysis, and screened the key gene P38. From the neutrophil level, targeting P38, we hope to provide a new solution for the treatment of ovarian cancer.

Declarations

Acknowledgements

The authors would like to acknowledge the scientific and technical assistance of the Tongji University School of Medicine, Shanghai, China.

Funding

This work was supported by the Fundamental Research Funds for the Central Universities (NO. kx0151620173399).

Authors' contributions

All of the authors participated actively in the experiments. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Specimens were collected and approved by the hospital ethics committee(SHSY-IEC-4.0/19-60/01).

Consent for publication

The patients were enrolled with informed consent.

Competing interests

The authors have declared that no competing interest exists.

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Figures

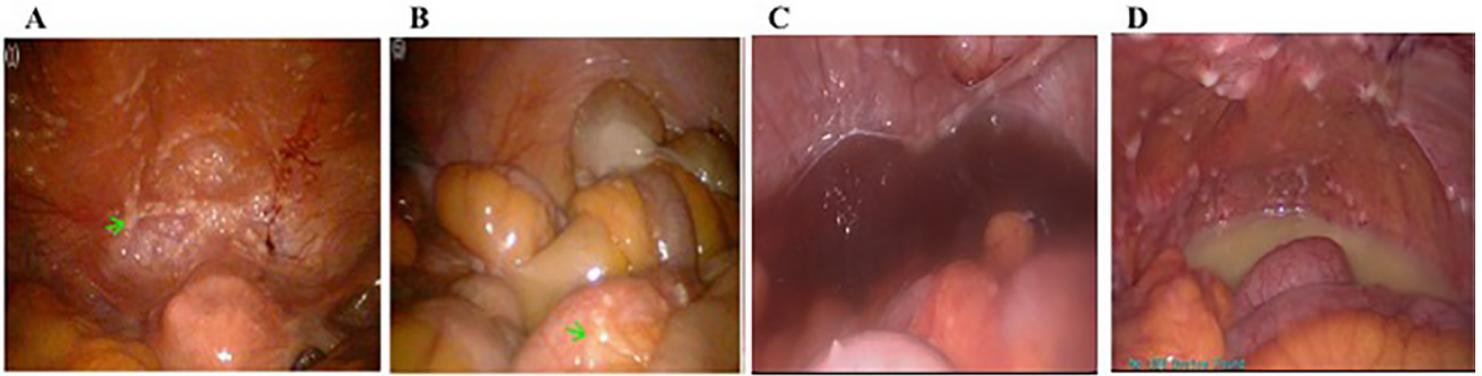


Figure 1

Clinical features of ovarian cancer metastasis: ovarian cancer metastasis is common in the peritoneum (A), intestine surface (B). Late ovarian cancer often shows ascites (C) and similar purulent infection (D)

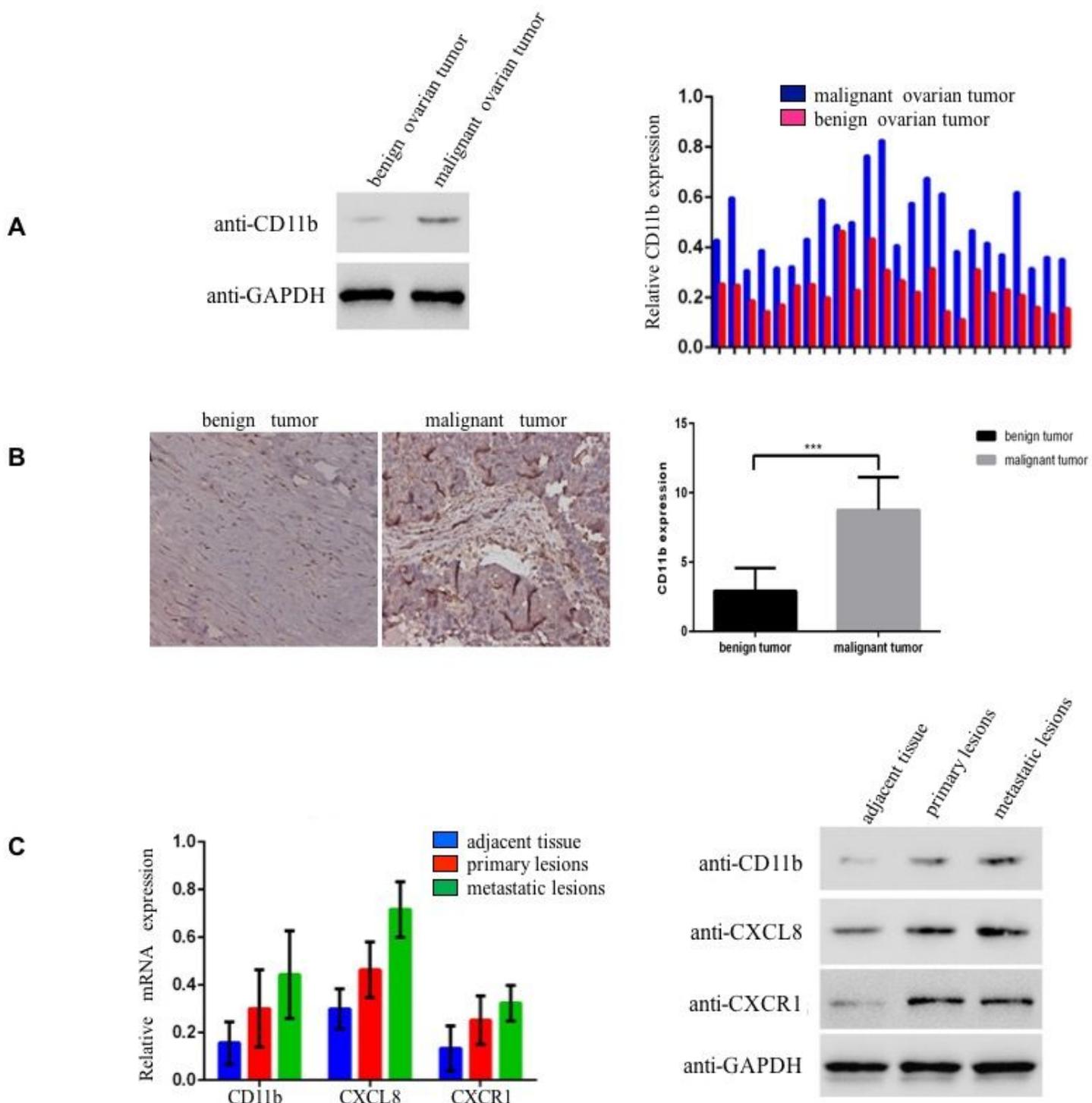


Figure 2

Neutrophils infiltration with ovarian cancer: (A) Protein expression level of CD11b was tested in 24 pairs of benign and malignant tumors tissues, Gray values displayed in the histogram. (B) CD11b expression of benign and malignant tumor tissues by immunohistochemistry. (C) Detection of neutrophil-related markers (CD11b, CXCL8, CXCR1) in ovarian cancer tissues (paracancerous, primary lesions, metastases) by PCR and WB.

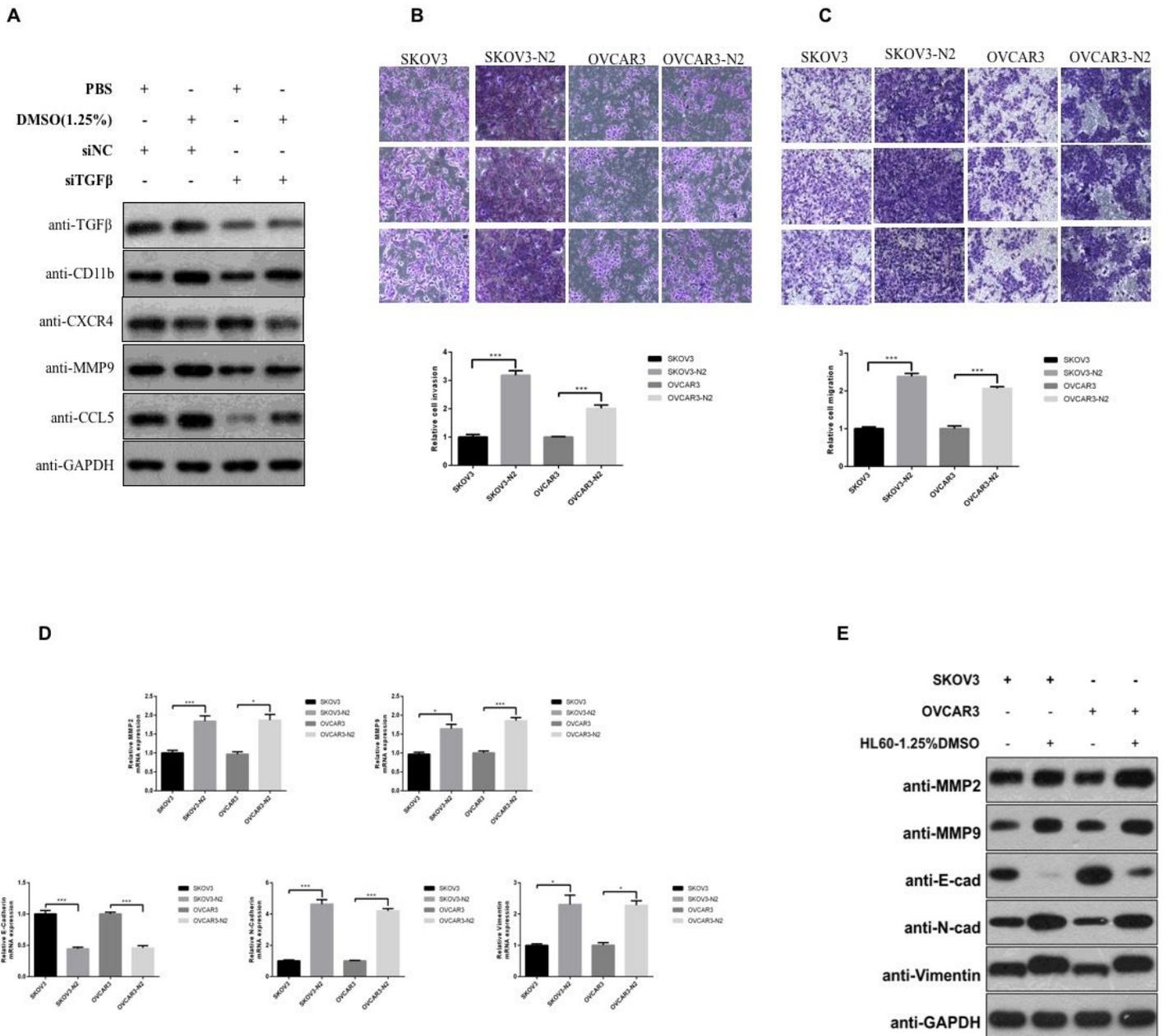


Figure 3

Construction of a tumor-associated neutrophil research model, demonstrating that N2 neutrophils promote ovarian cancer invasion and metastasis. (A) Detect the key indicators by the WB method to verify N1 and N2 HL60-siNC and HL60-siTGFβ were induced by 1.25% DMSO for 5 days. WB results suggest that both groups have increased CD11b expression. HL60-siNC induced by addition of 1.25% DMSO (N2) expressed high levels of CXCR4, MMP9, CCL5 than HL60-siTGFβ induced by the addition of 1.25% DMSO (N1). (B) Transwell experiment proves that N2 neutrophils promote ovarian cancer invasion. Invasive ability of ovarian cancer cells (SKOV3, OVCAR3) were increased after co-cultured with N2 neutrophils. (C) Transwell experiment proves that N2 neutrophils promote ovarian cancer metastasis. Metastasis ability of ovarian cancer cells (SKOV3, OVCAR3) were increased after co-cultured with N2 neutrophils. (D and E) Changes of metastasis-related indicators of ovarian cancer cells (SKOV3, OVCAR3)

after co-cultured with N2 neutrophils was assessed by PCR and WB. The expression of E-cadherin was significantly reduced after co-cultured with N2 neutrophils. While the expression of N-cadherin, Vimentin, MMP2 and MMP9 was significantly increased.

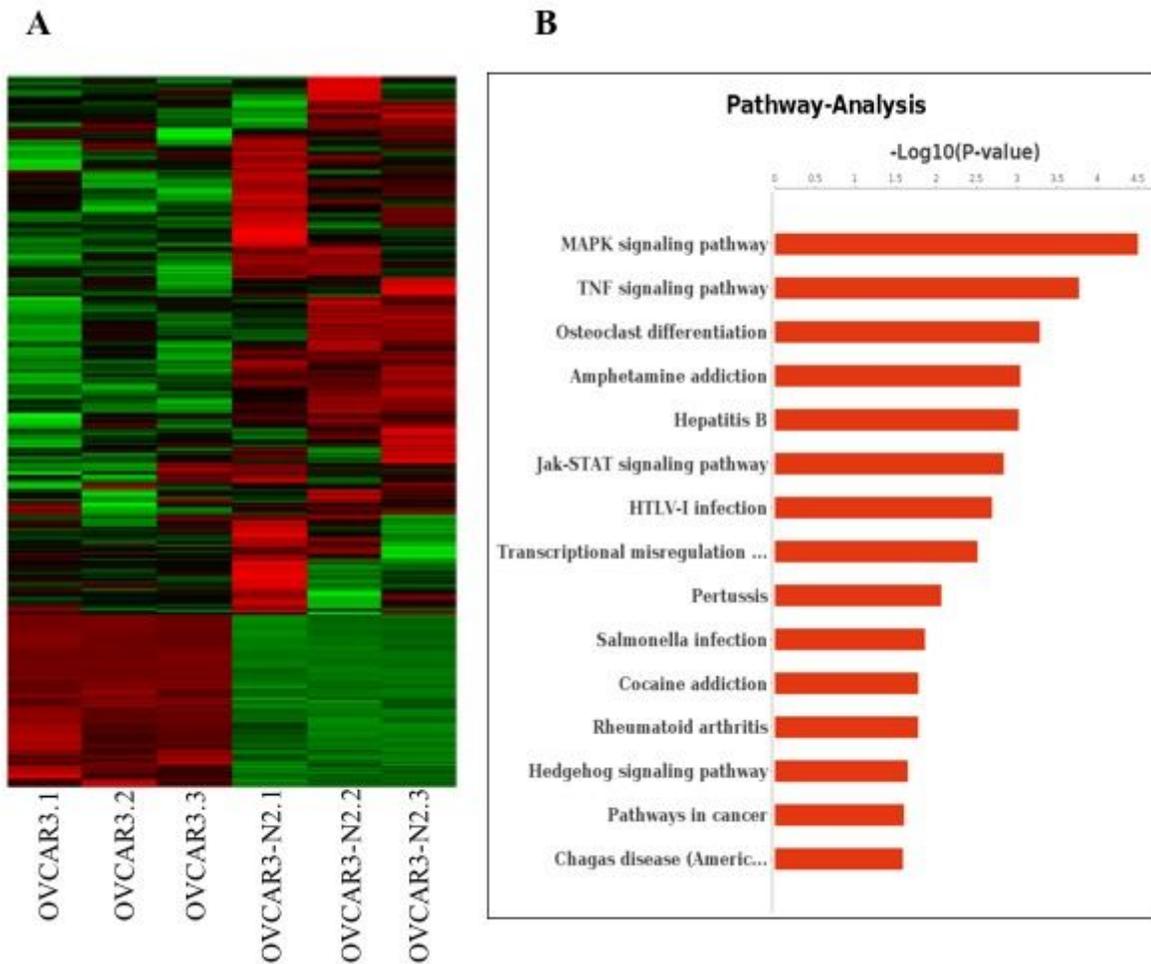


Figure 4

High-throughput sequencing seek for differentially expressed genes in the treatment group (OVCAR3-N2) and the control group (OVCAR3). (A) Gene heatmap was showed. Data analysis showed more than 700 differentially expressed genes, of which 73 were more than 3 times and 13 were more than 5 times. (B) Pathway analyses showed a significant up-regulation of MAPK signaling levels.

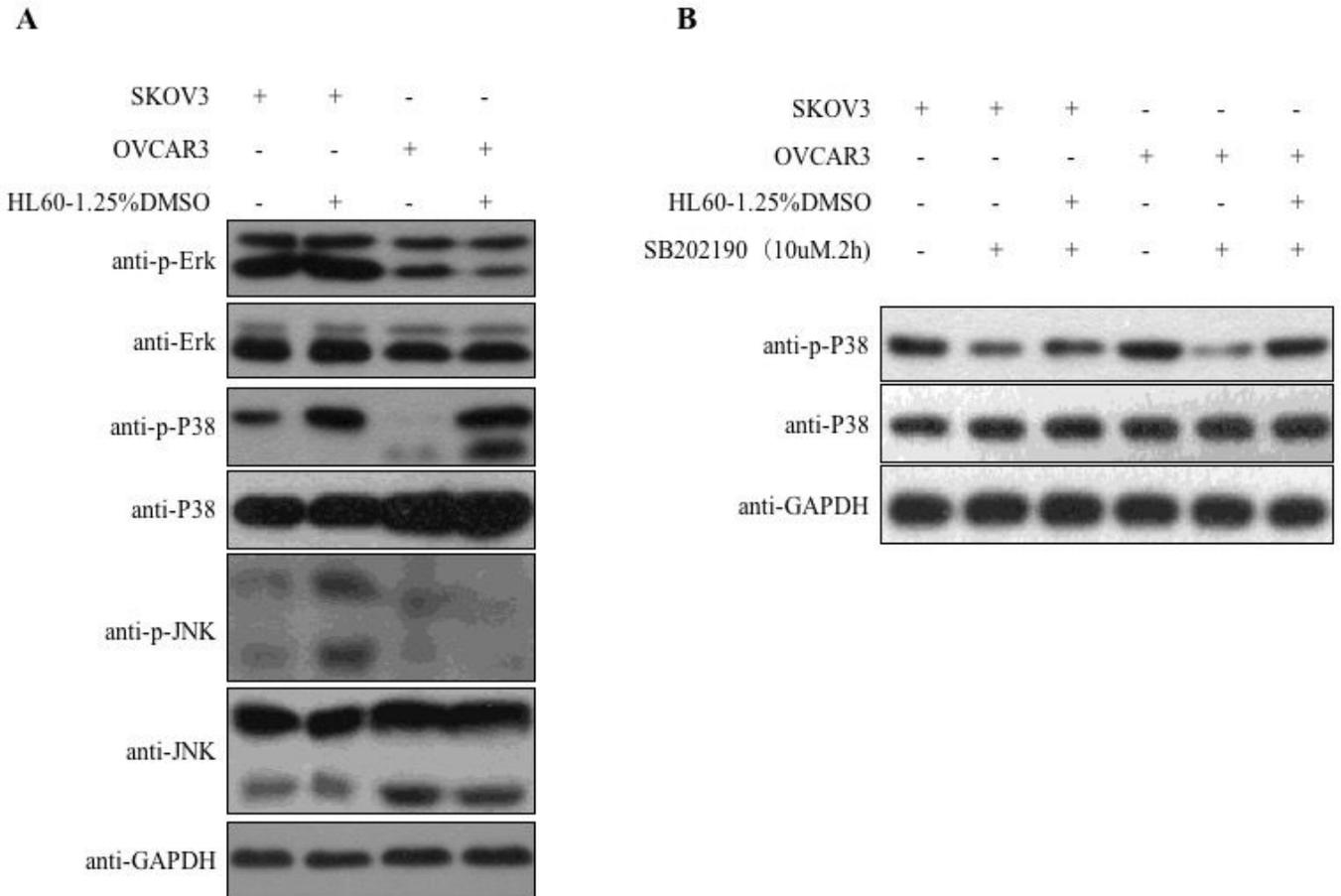


Figure 5

Search for key factors of MAPK signaling pathway.(A)Detect the different expression levels of key molecules in MAPK signaling pathway in treated and control cells. The expression level of p-P38 was significantly increased in SKOV3 and OVCAR3 cells after co-cultured with N2 neutrophils.(B)SB212090 can significantly inhibit the level of p-P38 in the control group (SKOV3, OVCAR3), but the inhibit effect in the experimental group (SKOV3-N2, OVCAR3-N2)were reduced.