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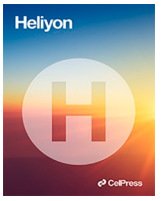

Research article

Ezrin's role in gastric cancer progression: Implications for immune microenvironment modulation and therapeutic potential

Yanli Zhu [a,b,c], Xue Zhang [a,b], Yi Chen [a], Qianli Liu [a], Jin Yang [a,b], Xiaoxiao Fan [a,b], Hanjun Song [a,b], Zhuoxin Cheng [d,*], Shuang Liu [a,b,**]

[a] Jiamusi University School of Basic Medicine, Jiamusi 154007, China
[b] Key Laboratory of Microecology-immune Regulatory Network and Related Diseases, Jiamusi 154007, China
[c] Digestive Disease Center, The First Affiliated Hospital of Jiamusi University, Heilongjiang Province, Jiamusi 154000, China
[d] Department of General Surgery, The First Affiliated Hospital of Jiamusi University, Heilongjiang Province, Jiamusi 154000, China

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ABSTRACT

At present, surgical resection is the most effective method for the treatment of gastric cancer. However, death caused by inoperable metastasis is still very common, despite research in this area. The mechanisms underlying the occurrence, development, and metastasis of gastric cancer are not fully understood. Ezrin, a plasma membrane-microfilament junction participates in a variety of cellular activities and is closely related to tumorigenesis and development. Few studies have explored the relationship between the tumor immune microenvironment and ezrin expression in gastric cancer. In this study, we used proteomic techniques to analyze the differentially expressed proteins between the gastric cancer cell lines MKN-45 and HGC-27 and screened ezrin as the target protein. We collected patient information from The TCGA and GEO databases, and the results showed that ezrin was positively correlated with adverse clinical features. We further explored the relationship between ezrin expression levels, immune microenvironment, and genomic changes. We found that ezrin was involved in immune regulation and genomic instability in gastric cancer. When the expression of ezrin is high, immune cell infiltration also increases. We also predicted that ezrin is closely related to immunotherapy and chemosensitivity. Single-cell transcriptome data showed that the ezrin gene was mainly expressed in B cells and epithelial cells, and the expression of EZR in these epithelial cells was positively correlated with the epithelial-mesenchymal transformation pathway and Pi3k-AKT pathway score. Through functional verification of the stably transfected cell line constructed by lentivirus, the results of the liver metastasis model in nude mice suggested that high expression of ezrin leads to the formation of more metastatic foci. In summary, our results clarify the prognostic, immunological, and therapeutic value of ezrin in gastric cancer and provide a theoretical basis for more accurate treatment.

* Corresponding author. Department of General Surgery, The First Affiliated Hospital of Jiamusi University, Heilongjiang Province, Jiamusi 154000, China.
** Corresponding author. Jiamusi University School of Basic Medicine, Jiamusi 154007, China.
E-mail addresses: czx6892551@yeah.net (Z. Cheng), lius@jmsu.edu.cn (S. Liu).

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1. Introduction

Gastric cancer is one of the most common malignant tumors worldwide [1]. According to statistics from the World Health Organization, over one million people are diagnosed with gastric cancer every year, and about 700,000 of them die from the disease [2]. The incidence of gastric cancer varies significantly across different regions, with the highest rates observed in Asian countries, particularly China, Japan, and Korea [3].

Epidemiological studies of gastric cancer mainly focus on several aspects, including population characteristics, environmental factors, genetic factors, and pathological types, among others [4]. In terms of population characteristics, research has shown that the risk of gastric cancer is higher in males, older individuals, those with a lower socioeconomic status, and certain occupation groups such as farmers and miners. Environmental factors have also been found to be closely associated with the development of gastric cancer, such as dietary habits (high-salt, high-fat diet), smoking, and alcohol consumption, all of which are considered potential risk factors for gastric cancer [5,6].

In addition, genetic factors play an important role in the occurrence of gastric cancer. Studies on familial gastric cancer have found that specific gene mutations may increase the risk of developing gastric cancer [7]. For example, in some cases of familial gastric cancer, there is an association between mutations in the BRCA1 and BRCA2 genes and the occurrence of gastric cancer [8].

Pathological type is another aspect that needs to be emphasized. Gastric cancer can be classified into two types: non-invasive and invasive. Non-invasive gastric cancer is usually detected earlier, while invasive gastric cancer tends to be more hidden. In-depth research on the pathological types of gastric cancer can help improve the accuracy of early diagnosis and treatment effectiveness [9]. Although epidemiological research on gastric cancer has made some progress, further exploration of its mechanisms and risk factors is needed to improve prevention, early diagnosis, and treatment measures for gastric cancer.

Ezrin belongs to the Ezrin-Radixin-Moesin (ERM) family and plays an important role in cell morphological changes and cell movement [10]. Recent studies have found that the expression of Ezrin in tumors is related to the development and metastasis of malignant tumors [11]. Ezrin is a transmembrane protein that connects the extracellular matrix with the intracellular cytoskeleton. It acts as a bridge between the cell membrane and the cytoplasm, regulating cell morphology and movement [12]. Ezrin can affect the proliferation of tumor cells by regulating the cell cycle and apoptosis pathways. Studies have found that overexpression of Ezrin is associated with increased proliferative activity of tumor cells, while inhibition of Ezrin can suppress the proliferation of tumor cells. High expression of Ezrin is also associated with increased invasive and metastatic abilities of tumor cells. Research has shown that Ezrin can regulate the reorganization of the cell cytoskeleton, enabling tumor cells to acquire invasive abilities and participate in maintaining the migratory capabilities of tumor cells [13]. Based on the important role of Ezrin in tumors, researchers have begun to explore the possibility of targeting it for therapy. Currently, some studies have reported the design and development of Ezrin inhibitors as potential therapeutic targets, with the hope of providing new avenues for tumor treatment [14]. However, the exact mechanisms of Ezrin in gastric cancer are still not fully understood, and further research is needed to elucidate its regulatory mechanisms and explore more effective treatment strategies.

Immunotherapy has made significant progress in tumor research, and new immunotherapies such as immune checkpoint inhibitors, CAR-T cell therapy, and tumor vaccines have brought new hope for the treatment of cancer patients [15,16]. However, there are still challenges, such as treatment resistance and side effects [17,18]. To better apply immunotherapy, further exploration of the mechanisms of immunotherapy, optimization of treatment regimens, and strengthening of monitoring and management of side effects are needed in future research. In this study, we aim to explore the role of Ezrin in the immune microenvironment of gastric cancer using public databases, providing a solid theoretical basis for immunotherapy of gastric cancer.

Gastric cancer, a predominant malignancy of the digestive system, presents with high incidence and mortality rates globally [19]. While various factors contribute to its pathogenesis, the mechanisms of cell death, particularly non-apoptotic cell death, have garnered significant attention in recent years. Unlike apoptosis, which is a programmed form of cell death, non-apoptotic cell death encompasses diverse pathways such as necroptosis, ferroptosis, pyroptosis, and autophagy, each with distinct biochemical and morphological characteristics. These non-apoptotic pathways play crucial roles in determining tumor progression, response to therapies, and the modulation of the tumor microenvironment. The intricate balance between apoptotic and non-apoptotic cell death can influence the fate of tumor cells, impacting disease progression and therapeutic outcomes. Within this context, our research delves into the role of Ezrin, a molecule implicated in tumorigenesis and tumor progression, and its potential interplay with these non-apoptotic cell death pathways in the landscape of gastric cancer.

2. Materials and methods

2.1. Collection of clinical samples

From 2021 to 2023, a total of 62 samples of non-metastatic primary gastric cancer tissues along with matched peritumor gastric cancer tissues were obtained from individuals at the First Affiliated Hospital of Jiamusi University in China. Additionally, serum samples were collected from both patients and healthy individuals. All protocols and procedures were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Jiamusi University (ID: 2023-600-02), and informed consent was obtained from all patients before the initiation of the study.

2.2. Plasma collection procedure

Blood samples were obtained from patients with gastric cancer and healthy volunteers. Venous blood was collected using 5-mL blood vials containing 3.2% sodium citrate. Subsequently, the blood samples were centrifuged at 1200 rpm for 10 min. The upper plasma was carefully separated and transferred into 1.5-mL EPP tubes, which were then stored in a freezer set at −20 °C until further use in the experiments.

2.3. Scratch experiment

To conduct the scratch experiment, a sterile 6-hole plate was prepared, and a horizontal line was drawn evenly behind the plate using a marker pen. Each hole was passed through three lines to ensure accurate photo positioning later on. Previously treated gastric cancer cell lines were then evenly inoculated into the wells, with approximately 5×10^6 cells in each well. The following day, using a 200-μL pipette tip, scratches were made on the cell layer perpendicular to the drawn line on the back of the plate from the previous day. The cells were then washed three times with sterile PBS to remove any detached cells. A serum-free medium was added to each well and the cells were cultured in an incubator. At specific time points (0, 24, 48, and 72 h), the cells were observed and photographed under a microscope. The cell migration ability was subsequently calculated using ImageJ software.

2.4. Immunohistochemical staining (IHC)

The gastric cancer tissues and corresponding paracancerous tissues were used for immunohistochemical staining (IHC). The sections were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol. After antigen retrieval with citric acid buffer, the slices were treated with 3% H2O2 to block endogenous peroxidase activity. The primary antibody was incubated with the slices overnight at 4 °C. The secondary antibody, labeled with horseradish peroxidase (HRP), was applied, followed by staining with 3magin3-diaminobenzidine chromogenic solution (DakoEnVision). Hematoxylin was used for counterstaining. The slides were sequentially immersed in different concentrations of alcohol for dehydration. A neutral resin was used to seal the slides, which were then covered with a glass slide and allowed to dry. The photographs were captured using a microscope.

2.5. Immunofluorescence staining

The gastric cancer cell lines, HGC-27 and MKN-45, were inoculated into 24-well plates, and the experiment was carried out after the cell adhesion growth density reached 80%. The medium was removed from the 24-well plate, the cells were washed with PBS three times, after which 4% paraformaldehyde was added (1 mL per well) for 20 min to fix the cells. The samples were then placed in paraformaldehyde and washed three times with PBS (5 min each time). The samples were then sealed with 10% goat serum homologous to the secondary antibody (prepared in PBS) for 2 h. Diluted anti-EZR (1 AF594 1000) was incubated overnight at 4 °C, the first antibody was absorbed, and the samples were washed in PBS three times. Diluted AF594 antibody was then added and the culture allowed to incubate for 60 min. The nucleus was stained with DAPI and photographed by confocal microscope after sealing.

2.6. Animal model

BALB/c nude mice were purchased from Weitong Lihua Co. Ltd. All mice were anesthetized with inhalation before operating. The mouse liver metastasis model, 75- 125ul of HGC-27/MKN-45 cell suspension was injected into the spleen. After ligating the blood vessels around the spleen, the spleen was severed. Tumor tissues were collected for follow-up experiments.

2.7. Survival analysis

The data analyses were conducted using the RNAseq data (level 3) and corresponding clinical information of GC from the cancer genome map (TCGA) dataset available at https://portal.gdc.com. The R software package survival and survminer were used for this analysis. The KM survival curve was generated to compare the survival distribution of different groups of samples in the TCGA dataset. The log-rank test was used to assess the differences between the groups, and the 95% confidence interval (CI) represented the hazard ratio (HR) confidence interval. The median survival time (Medaintime) in years was determined as the corresponding time at which 50% of the samples in each group survived.

2.8. TMT quantitative proteomic analysis

This analytical procedure was performed with the assistance of Hangzhou Jingjie Biotechnology Co., Ltd. To prepare the protein samples, when the cultured HUVECs density reached 90%, the cells were stimulated with 0.4 μg/mL NETs for 24 h and centrifuged at 12,000 g and 4 °C for 10 min, after which cell deposition was collected. There were three samples each in the stimulation group and three samples in non-stimulation group, and the number of cells in each sample exceeded 5 million. The collected fine cell precipitates were stored at −80 °C for TMT and PRM experiments.

The isobaric analysis was based on relative and absolute quantitative (iTRAQ) proteomics. By searching the UniProt database, the functions of numerous kinds of protein was obtained. The following thresholds were used: $P < 0.05$ and 1.2 times. All differentially

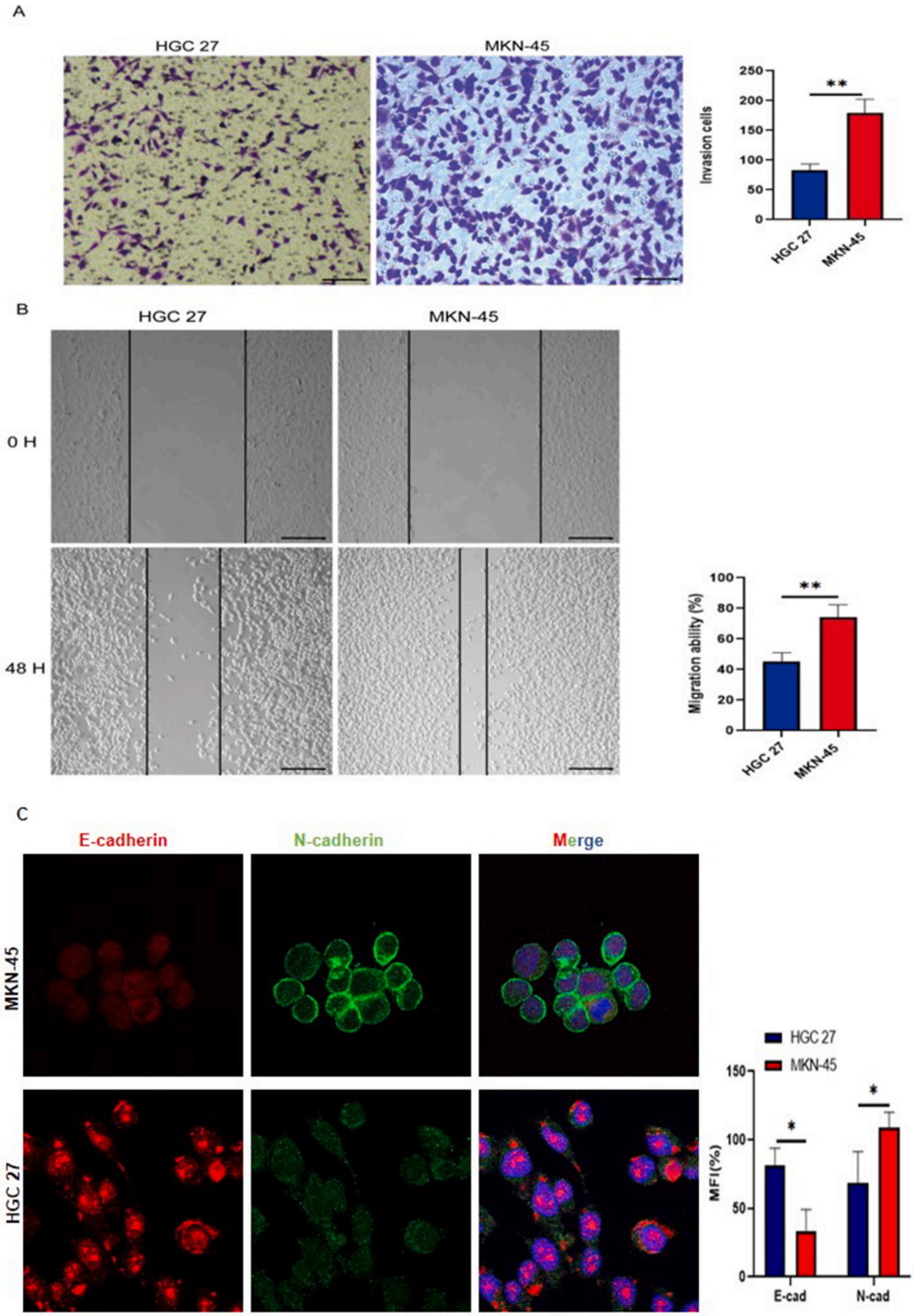

Fig. 1. The invasion and metastasis ability of metastatic gastric cancer cell line MKN-45 is stronger than that of in situ gastric cancer cell line HGC -27 A. Transwell test verified the invasive ability of MKN-45 and HGC -27 B. Scratch test verifies the migration ability of MKN-45 and HGC-27 C. Analysis of E-cad and N-cad expression levels in MKN -45 and HGC -27 by immunofluorescence staining.

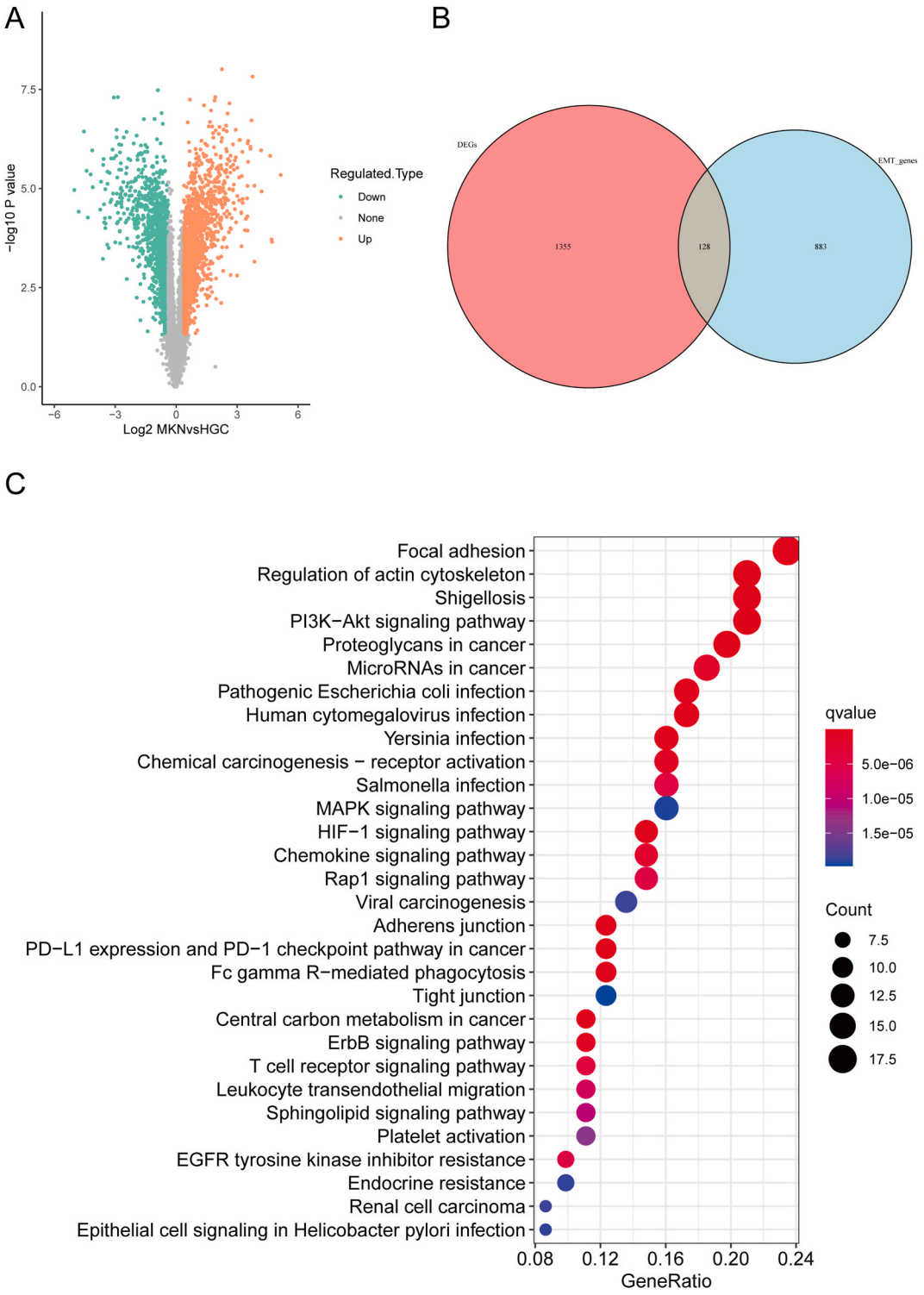

Fig. 2. Proteomic techniques reveal the heterogeneity of gastric cancer cells A. Differential protein volcano chart B. The up-regulated differential protein and EMT data set were taken to overlap C. Enrichment Analysis of differential proteins by KEGG.

expressed proteins were selected for enrichment analysis using a heat map and the Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.9. Single-cell clustering analysis

We carried out ScRNA-seq analysis on 10 newly acquired human tissue samples obtained from 6 patients. These samples included 3

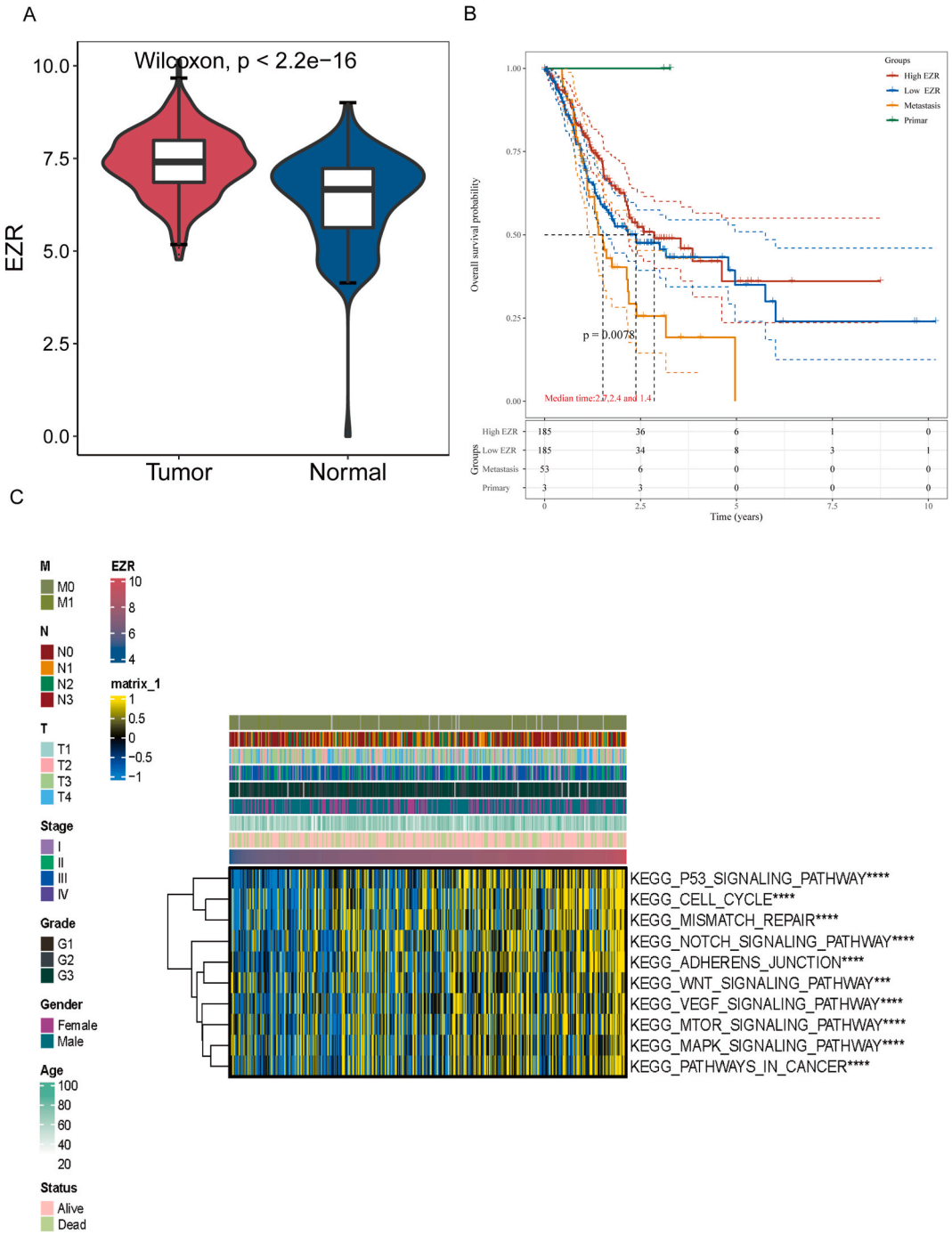

Fig. 3. Analysis of the correlation between the expression level of Ezrin and clinical features of gastric cancer A. The expression level of Ezrin in tumor tissue and corresponding normal tissue B. KM Survival Curve Distribution of different groups of samples (Ezrin High expression Group, Ezrin low expression Group, Cancer in situ Group, and liver Metastasis Group) C. Analysis of Ezrin expression level, clinicopathological characteristics, and tumor-related signal Pathway Activation in TCGA data set.

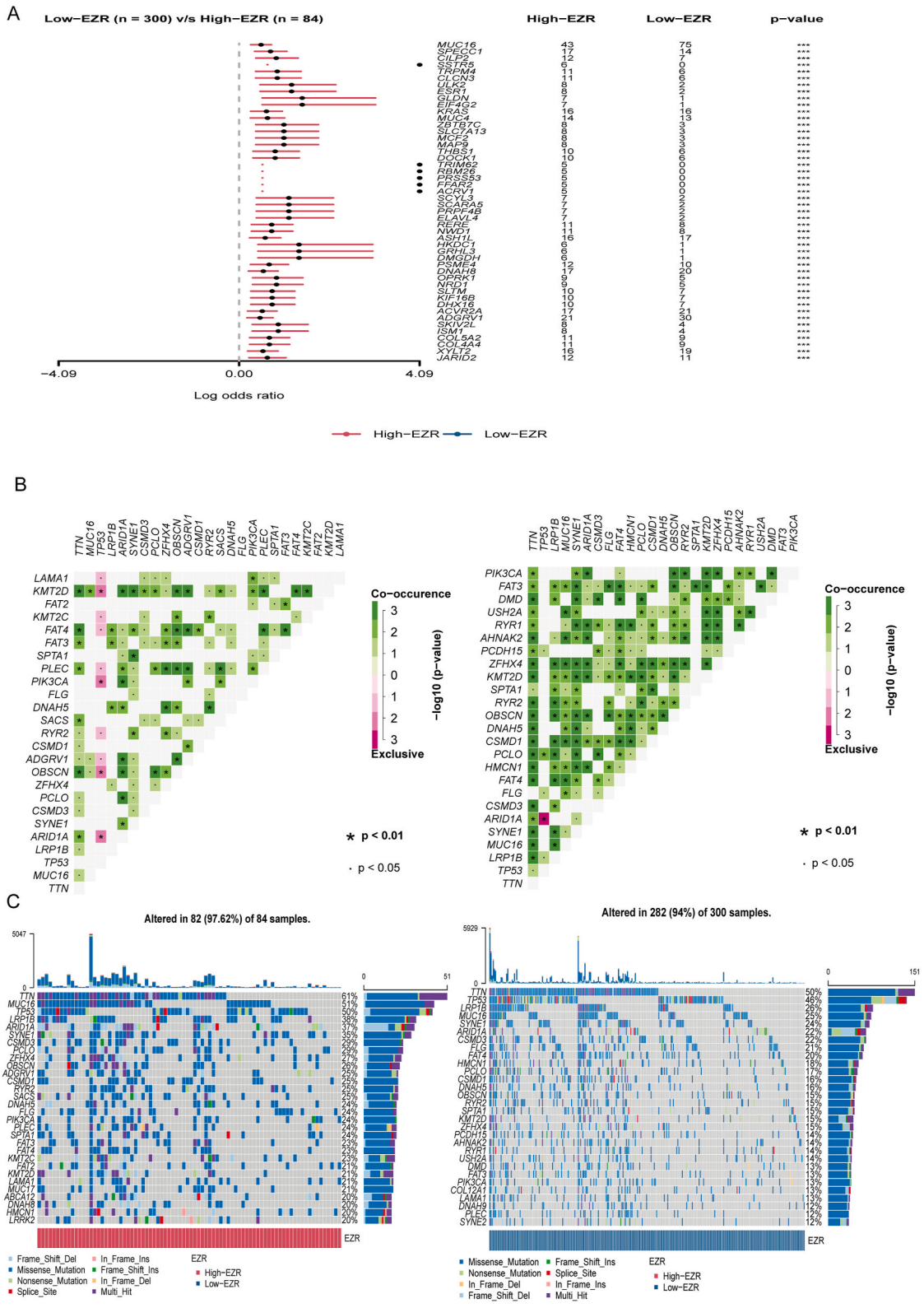

Fig. 4. Analysis of the correlation between Ezrin expression level and tumor microenvironment genomic instability A. Comparison of gene mutation load between high and low Ezrin expression groups. B. Gene mutation heat map illustrating differences between high and low Ezrin expression groups. C. Overall distribution landscape of gene mutations in high and low Ezrin expression groups.

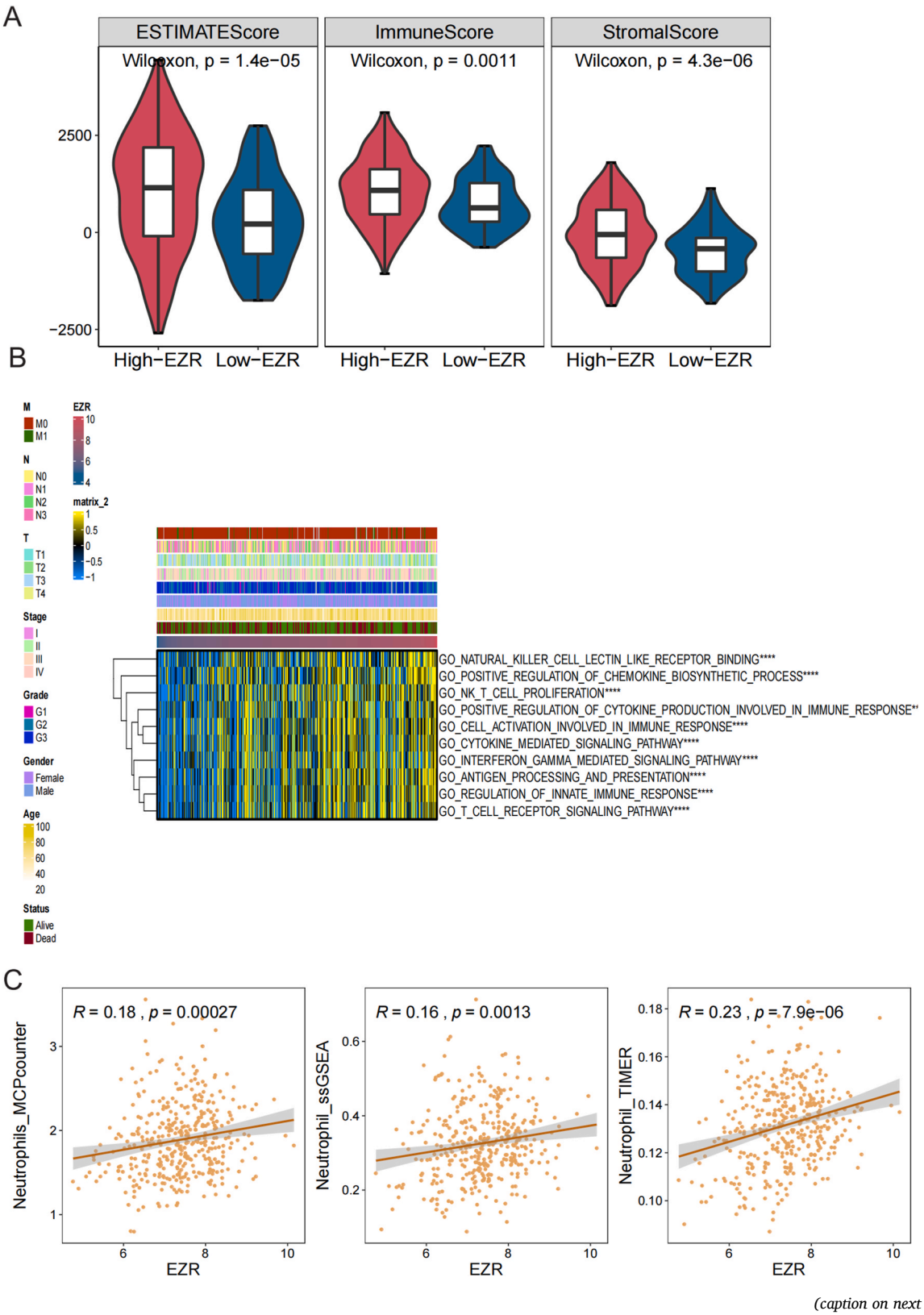

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Fig. 5. Analysis of the correlation between Ezrin expression level and gastric cancer immune microenvironment A. Relationship between Ezrin expression level and ESTIMATE score, immune score, and stromal score. B. Relationship between Ezrin expression level and clinicopathological features and activation of immune-related signaling pathway. C. Correlation between Ezrin estimated by three types of immune infiltration and neutrophil infiltration.

primary tumor samples (designated as PT1, PT2, and PT3), one adjacent non-tumor sample (designated as NT1), and 6 metastatic samples (MT). After conducting quality control measures (with a threshold of nFeature_RNA >200), we ultimately obtained 42,871 cells with 11,327 transcripts.

2.10. Collection of non-apoptotic cell death-related gene sets

Non-apoptotic cell death-related genes were collected from published studies (10.1016/j.ijsu.2022.106936) [20].

2.11. Data analysis

The data presented in this study are represented as mean ± standard deviation (SD). Statistical analysis was performed using SPSS software version 22.0. The *t*-test was conducted to determine the differences between the groups. A value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Metastatic gastric cancer cell lines showed a stronger ability for migration, invasion, and epithelial-mesenchymal transformation

The results of the Transwell and the scratch test showed that MKN-45 had a stronger ability to migrate and invade than HGC-27 (Fig. 1A and B). At the same time, we used immunofluorescence staining to label the representative protein N-cad and Vimentin of EMT and found that the expression level of MKN-45 was higher (Fig. 1C). The above results suggest that our cell lines from different sources have different biological characteristics, and the mechanism is worth exploring.

3.2. Unveiling the diversity of gastric cancer cells through proteomic techniques

Tandem mass spectrometry tags (TMT) -based quantitative proteomics is a highly robust method for analyzing differentially expressed proteins (DEPs). It boasts the highest throughput, minimal systematic errors, and remarkable functional capabilities. With its qualities of sensitivity, separation ability, and throughput, TMT quantitative proteomics was employed to investigate intra-tumor heterogeneity by comparing the differential proteins between gastric cancer cell lines MKN-45 and HGC-27. The analysis revealed that 1484 proteins were upregulated while 1434 proteins were downregulated in MKN-45/HGC-27 (Fig. 2A). As tumor cell metastasis is primarily associated with epithelial-mesenchymal transition (EMT), we intersected the upregulated proteins with EMT-related genes in the TCGA database, resulting in 128 proteins (Fig. 2B). These 128 differential proteins were subjected to functional analysis (Fig. 2C–Table S1) to gain insight into their biological role.

3.3. The expression of Ezrin showed a positive correlation with the unfavorable clinical features of gastric cancer

By utilizing the TCGA and GTEx databases, it was determined that gastric cancer tissue exhibited higher levels of Ezrin expression compared to normal tissue, confirming its overexpression in tumors (Fig. 3A). RNAseq data (level 3) and accompanying clinical information on gastric cancer were sourced from the TCGA dataset (https://portal.gdc.com). Analysis revealed that patients with distant metastasis and high Ezrin expression experienced shorter survival times (Fig. 3B). In gastric cancer, elevated levels of Ezrin transcription were associated with more unfavorable clinicopathological features, including advanced clinical stages, pathological grades, and activation of pathways linked to tumor progression (Fig. 3C). These findings indicate a strong association between increased Ezrin levels and disease progression.

3.4. The expression level of Ezrin has an impact on the genomic instability of the tumor microenvironment

Cancer is characterized by genomic instability and mutations. This instability can be observed in various malignant tumors and precancerous lesions and is also associated with drug resistance. Using Fisher's exact test, we determined the mutation frequency ratio between the high and low Ezrin groups and sorted them based on increasing p values. Compared to the high Ezrin group, the low Ezrin group had extremely low mutation loads for CILP2 and SSTR5, while having higher mutation loads for MUC16 and ADGRV1 (Fig. 4A). We then obtained a correlation heat map showing the relationship between different gene mutations, with colors representing the corresponding P values, and marking those with $P < 0.05$ and $P < 0.01$ (Fig. 4B).

The overall distribution of mutations revealed that TTN mutations were enriched in both the high and low Ezrin groups (61% and 53%, respectively) (Fig. 4C). MUC16 mutations were more common in the high Ezrin group (51%) and partially observed in the low Ezrin group (25%). In the high Ezrin group, the three most common mutations were TP53 (50%), LRP1B (38%), and ARID1A (37%). In

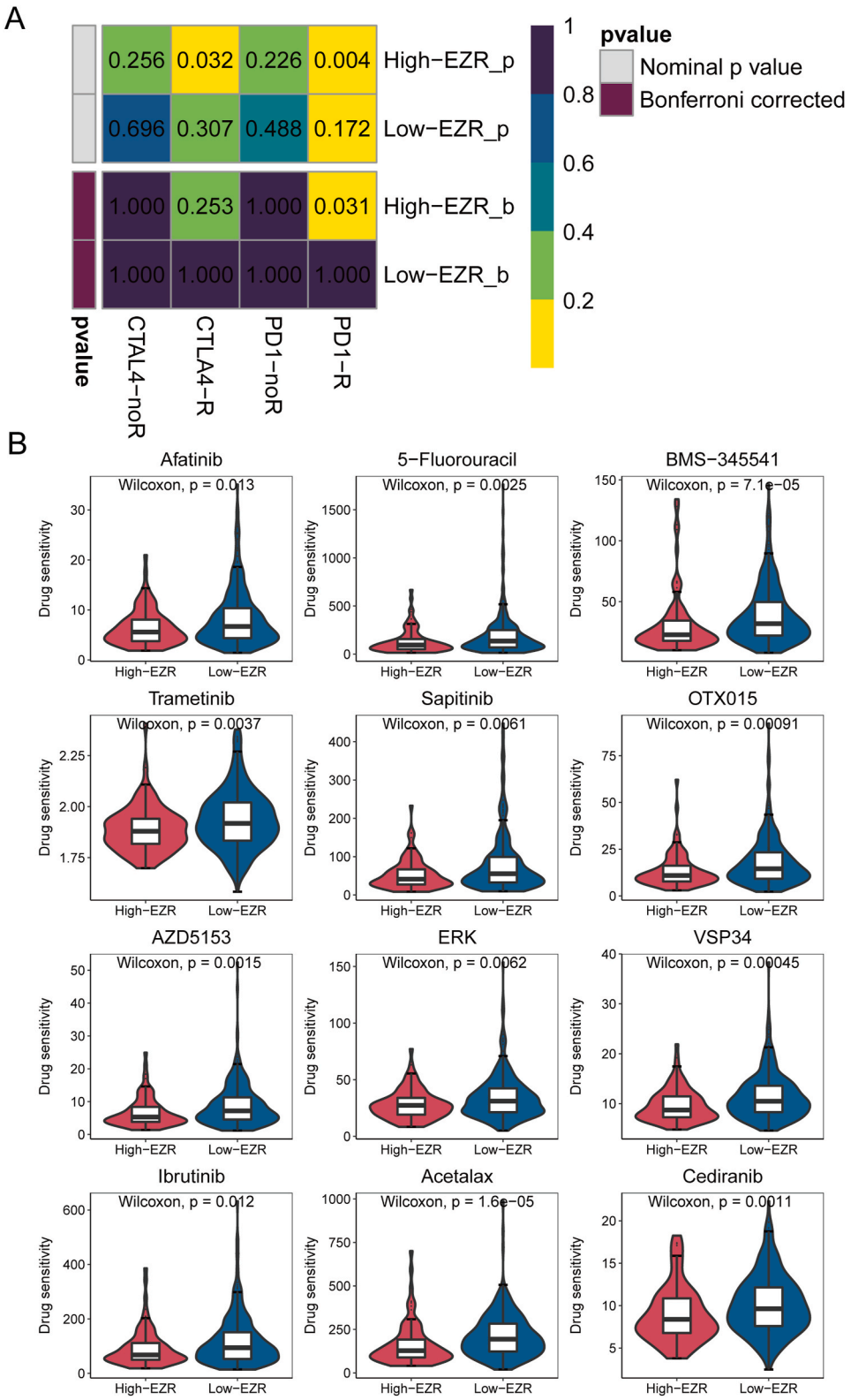

Fig. 6. Immunotherapy and chemotherapy response in TCGA-STAD based on Ezrin expression level A. Analysis of Ezrin expression level in TCGA-STAD and its correlation with immune checkpoint ability. B. Sensitivity analysis of common chemotherapeutic drugs in the high and low Ezrin expression groups.

the low Ezrin group, there was a significant mutation (Fig. 4C) of TP53 (46%).

3.5. The immune microenvironment of gastric cancer is regulated by the expression level of Ezrin

The tumor microenvironment includes immune cells, and their infiltration characteristics are determined using ESTIMATE, MCP counter, ssGSEA, and TIMER algorithms from the TCGA dataset. It is important to mention that there was a positive correlation between Ezrin expression and matrix score, immune score, and tumor purity (Fig. 5A). Furthermore, Ezrin expression showed significant correlations with various immune cell infiltrations, particularly noteworthy was the positive correlation with neutrophil infiltration (Fig. 5B and C).

3.6. The expression of Ezrin in patients with gastric cancer has an impact on their response to immunotherapy and chemotherapy

The advent of immunotherapy has provided new hope for cancer patients, particularly with the success of PD-1/PD-L1 monoclonal antibody drugs. This success has paved the way for tumor immune cell therapy, and immunotherapy is now considered a growing trend in cancer treatment. The findings in Fig. 6A demonstrate that the response to immunotherapy varies between the high Ezrin group and the low Ezrin group. Specifically, gastric cancer patients with high Ezrin expression are more likely to respond positively to immunotherapy (Fig. 6A), which is of significant importance for the accurate selection of patients suitable for this type of treatment. Additionally, we also assessed the differences in response to various chemotherapeutic drugs between the high Ezrin group and the low Ezrin group. The results indicated that the high expression group exhibited lower sensitivity to the following chemotherapeutic drugs: Afatinib, 5-Fluorouracil, BMS-345541, Trametinib, Sapitinib, OTX015, AZD5153, ERK, VSP34, Ibrutinib, Acetalax, and Cediranib compared to the low expression group (Fig. 6B). Therefore, targeting Ezrin may prove beneficial for a larger number of patients undergoing chemotherapy.

3.7. The expression level of Ezrin is crucial in determining the pattern of non-apoptotic cell death

Using the ssGSEA algorithm, we assessed the levels of 12 non-apoptotic cell death types in TCGA-STAD and investigated their correlation with Ezrin. Our analysis revealed a significant positive association between Ezrin and Pyroptosis, Ferroptosis, Necroptosis, Cuproptosis, Entotic cell death, and Alkaliptosis(Fig. 7).

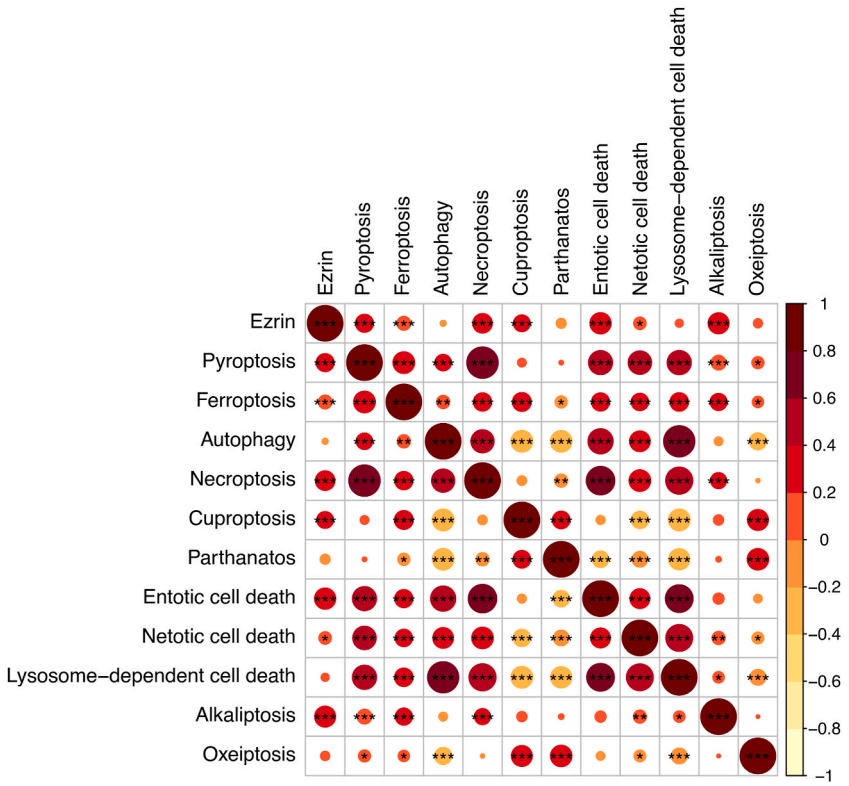

Fig. 7. Correlation between Ezrin and non-apoptotic cell death.

3.8. The comprehensive exploration of all cell types within the tumor microenvironment was achieved through the application of single-cell transcriptome analysis

In this study, we conducted a comprehensive landscape analysis of single-cell transcriptomes for all cell types in the tumor microenvironment. Fresh human tissue samples from 6 patients were subjected to single-cell RNA sequencing (scRNA-seq), including 3

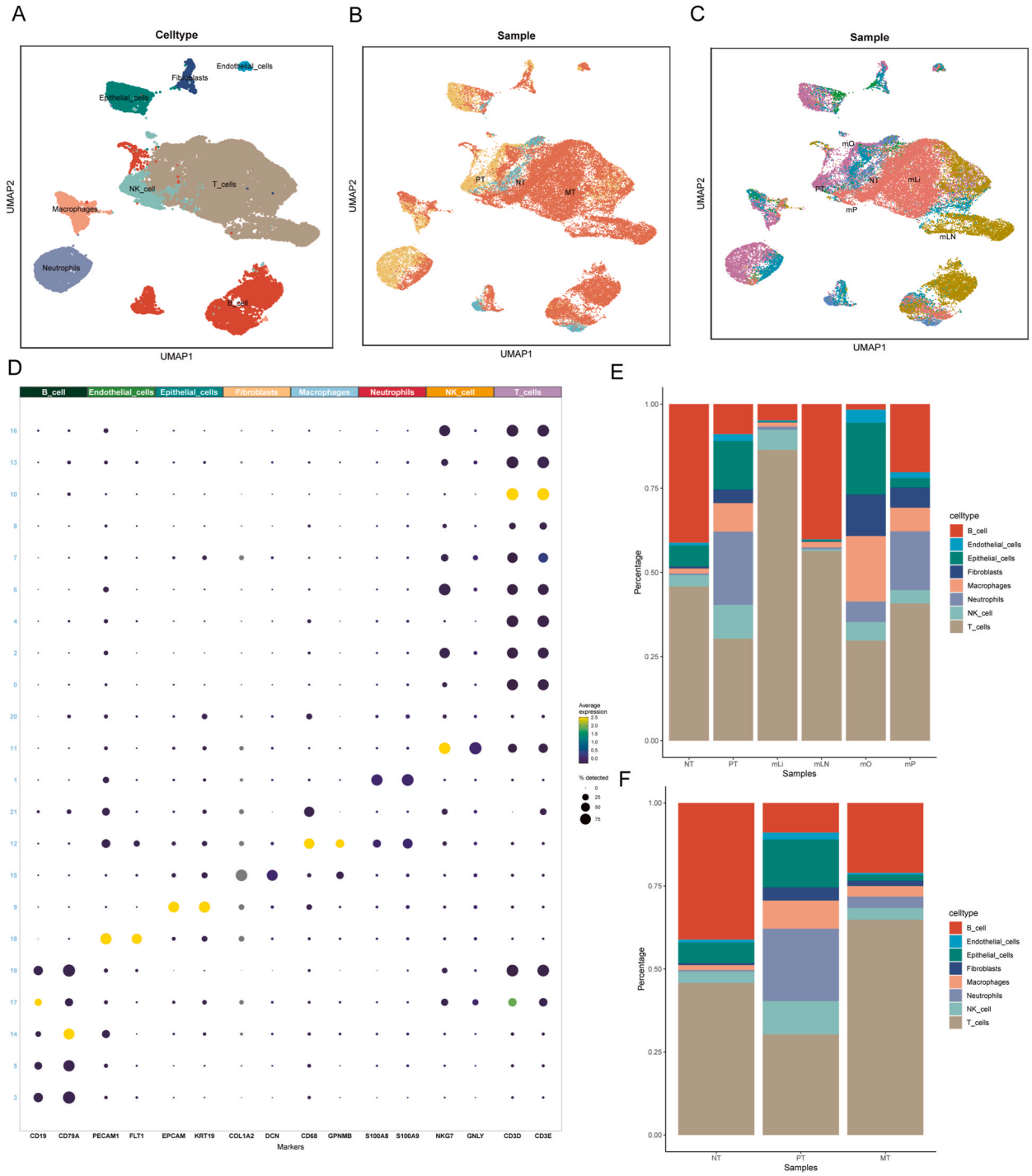

Fig. 8. Single-cell transcriptome analysis was conducted to investigate the general cellular composition of the tumor microenvironment A-C. Demonstrates the cell score observed in primary tumor samples, adjacent non-tumor samples, and metastatic samples. Additionally. D- F. Presents the distribution of cells in various samples.

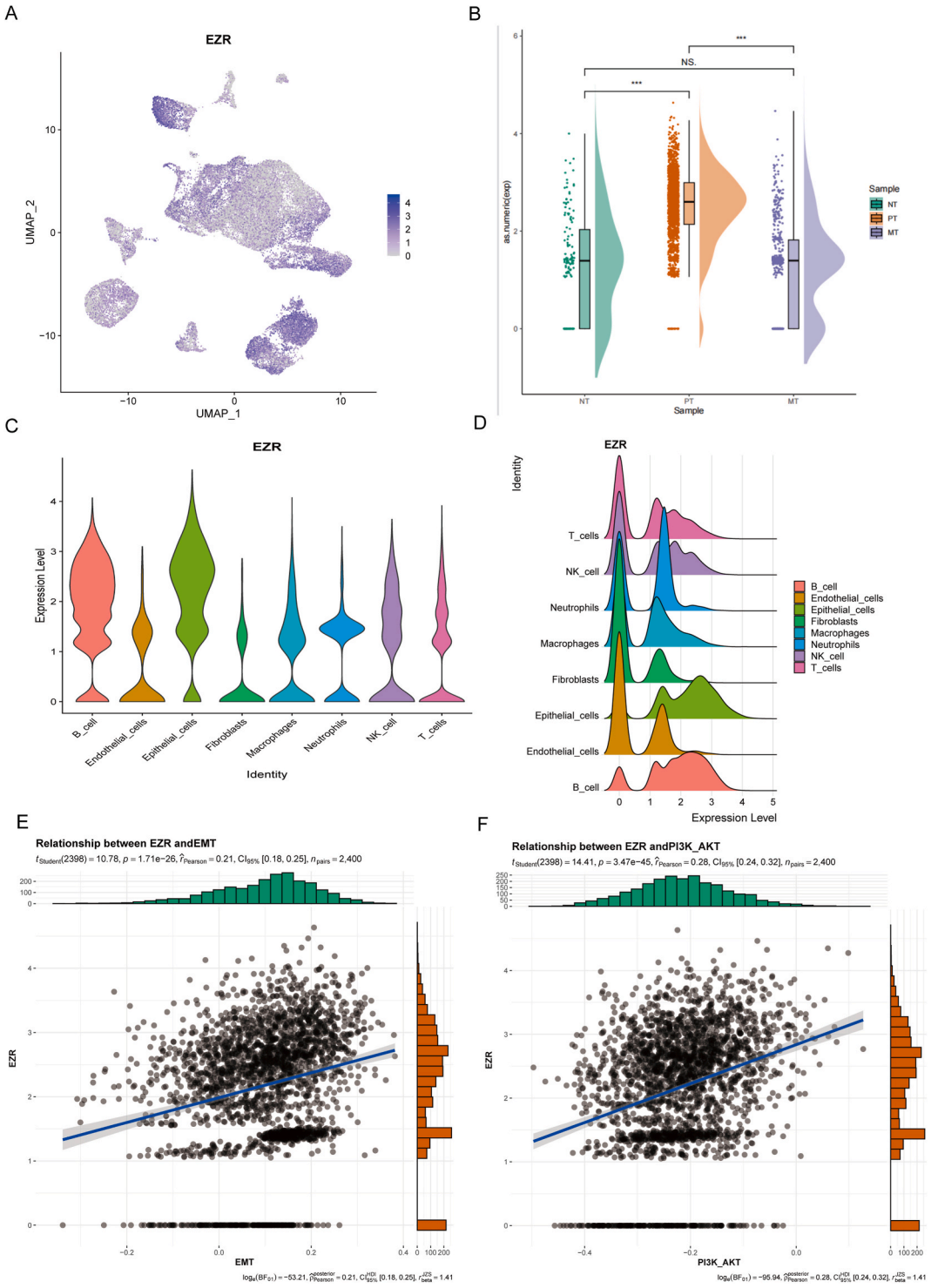

Fig. 9. Showcases the distribution of Ezrin in different cell types. A-D. The diagram presents the general landscape of Ezrin distribution, as well as the expression of Ezrin in normal tissues, orthotopic tumors, and metastatic tumors. Moreover, the figure displays the expression of Ezrin in various types of cells, including cancer cells, endothelial cells, fibroblasts, and epithelial cells E. Additionally, the research analyzes the relationship between EZR expression and the EMT pathway F. As well as the correlation between EZR expression and the PI3K-AKT pathway.

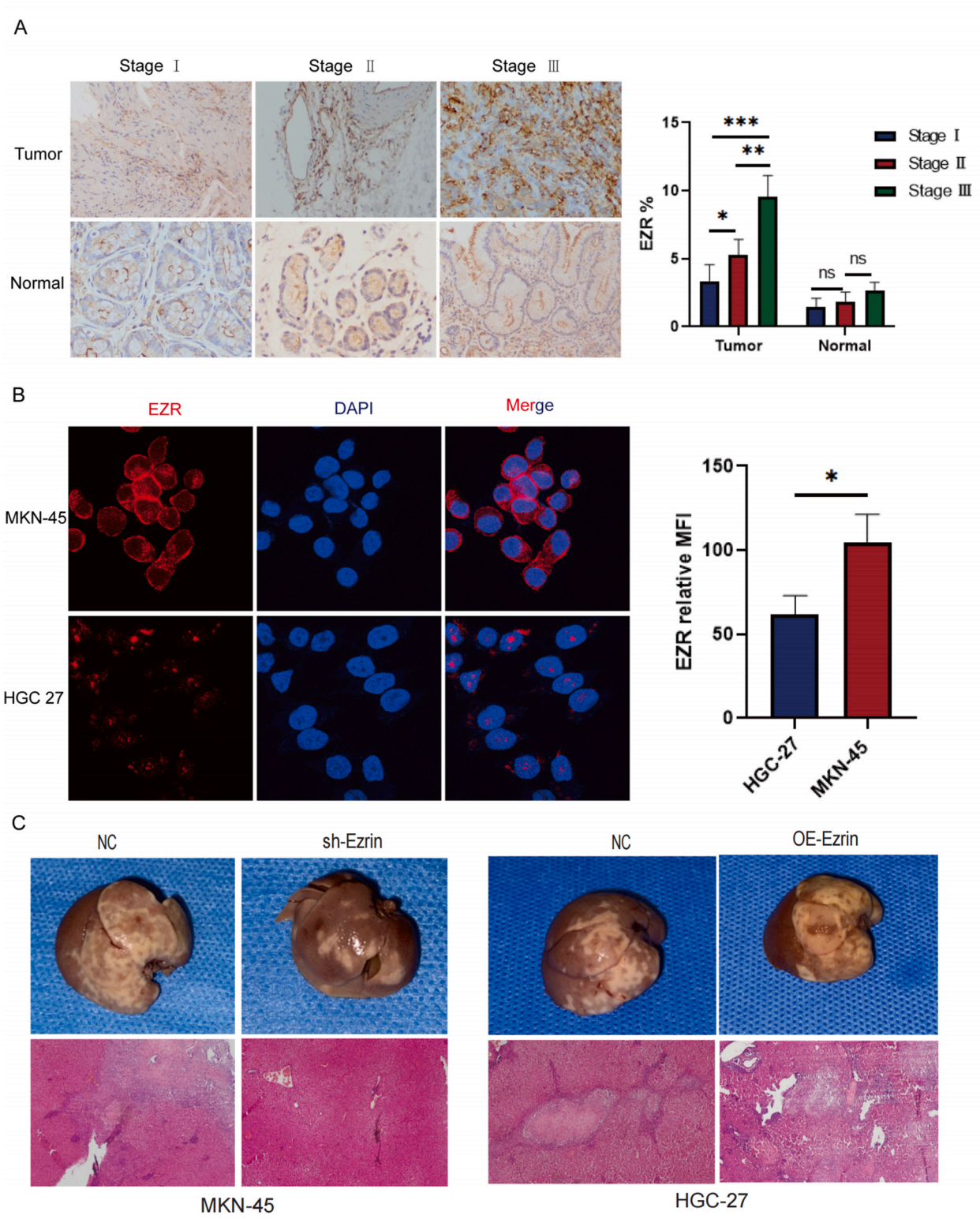

Fig. 10. Investigates the role of Ezrin in gastric cancer metastasis by analyzing its expression in clinical samples and cell lines. A. This was achieved through immunohistochemical detection of Ezrin in different stages of gastric cancer and corresponding paracancerous tissues, B. As well as by using immunofluorescence to detect its expression and localization in MKN-45 and HGC-27. C. Furthermore, an animal model was employed to demonstrate the impact of high and low Ezrin expression on the metastatic ability of gastric cancer cells.

primary tumor samples (PT1, PT2, and PT3), one adjacent non-tumor sample (NT1), and 6 metastatic samples (MT). After quality control (nFeature_RNA >200 & nFeature_RNA <5000, nUMIs >1000, transcripts mito <20), a total of 42,871 cells and 11,327 transcripts were obtained. The expression profiles were normalized using the SCTransform function in the R Seurat package, and the top 20 principal components were selected for dimensionality reduction. Clustering was performed with the resolution = 0.5 parameter. We identified B cells (CD19, CD79A), endothelial cells (PECAM1, FLT1), epithelial cells (EPCAM, KRT19), fibroblasts (COL1A2, DCN), macrophages (CD68, GPNMB), neutrophils (S100A8, S100A9), natural killer cells (NKG7, GNLY), and T cells (CD3D, CD3E) (Fig. 8A). There were significant differences in the proportions of these cell lineages between different primary tumors and metastatic tumors, revealing changes in cellular states at different stages of the disease (Fig. 8B–F).

3.9. Expression of Ezrin in B cells and epithelial cells

The analysis conducted using featurePlot and Vlnplot revealed that the Ezrin gene exhibited high expression primarily in B cells and epithelial cells (Fig. 9A–C, D). This finding raises the possibility of its involvement in promoting epithelial-mesenchymal transition, which warrants further investigation. Subsequently, we compared the expression levels of the EZR gene in orthotopic tumors, metastatic tumors, and normal tissues. The results demonstrated that EZR gene expression was highest in primary tumors, and its expression in the aforementioned epithelial cells (NT-142, PT-1736, MT-522) exhibited a positive correlation with the EMT pathway and pi3k-AKT pathway ($p < 0.05$) (Fig. 9B–E, F). These findings strongly suggest that Ezrin plays a significant role in driving tumor progression.

3.10. The expression of Ezrin significantly facilitates tumor progression, as evidenced by our findings

To further investigate its involvement in the development of gastric cancer, we utilized clinical samples to generate paraffin sections. The results of immunohistochemical staining revealed an increase in Ezrin production in tumor tissue with advancing clinical stage (Fig. 10A). To validate these histological results, we conducted immunofluorescence staining in gastric cancer cell lines MKN45 and HGC-27. Consistent with the histological findings, MKN-45 exhibited stronger fluorescence intensity compared to HGC-27, reaffirming the reliability of our histological results (Fig. 10B). Additionally, we established stable cell lines with knocked down and overexpressed Ezrin through lentivirus manipulation at the cellular level. By creating a liver metastasis model in nude mice, we demonstrated a positive correlation between Ezrin expression levels, gastric cancer cell metastatic ability, and metastatic focus (Fig. 10C). Therefore, our findings strongly suggest that Ezrin plays a vital role in promoting gastric cancer metastasis and has potential as a clinical therapeutic target.

4. Discussion

Based on the latest data from GLOBOCAN, the global incidence of gastric cancer in 2020 was 1.089 million cases, with an age-standardized incidence rate of 11.1 per 100,000 population, ranking it fifth among all malignant tumors [1,21]. Gastric cancer also ranked fourth in terms of mortality, with 769,000 deaths and an age-standardized mortality rate of 7.7 per 100,000 population, following lung, colorectal, and liver cancer. The heterogeneity of the immune microenvironment is a significant factor contributing to drug resistance, recurrence, and poor prognosis in cancer [22,23]. Recent advancements in chemotherapy, immunotherapy, and combination therapies have provided hope for patients with advanced cancer [24–26]. To better understand the tumor immune microenvironment, exploring its heterogeneity is crucial in treatment selection, predicting efficacy, developing combination treatment strategies, and identifying new immunotherapy targets.

In this particular study, we utilized proteomic techniques to analyze the differential proteins between metastatic gastric cancer cell line MKN-45 and in situ gastric cancer cell line HGC-27. Through this analysis, we identified Ezrin as the target protein and observed its significant association with adverse clinical outcomes in gastric cancer patients. By employing bioinformatics and machine learning methods, we discovered that Ezrin has a pronounced immunomodulatory effect on the gastric cancer microenvironment and a notable impact on genomic instability. The high expression of Ezrin makes it a promising target for immunotherapy. Furthermore, single-cell sequencing results revealed that epithelial cells express the Ezrin gene, indicating its potential role in inducing epithelial-mesenchymal transition, which warrants further investigation. Animal experiments demonstrated that inhibiting Ezrin expression significantly reduces metastasis incidence. Consequently, targeting Ezrin could represent a promising and effective treatment strategy in the future.

The Ezrin protein, a crucial member of the ERM family, has gained recognition in recent years [27]. Encoded by the Vi12 gene on chromosome 6q25.2, the mRNA Q26 has a length of 3166bp and results in a protein consisting of 585 amino acids. Initially isolated and purified from the brush border of chicken intestinal epithelial cells in 1981, Ezrin, through its association with the mediating membrane and cytoskeleton, influences cell morphology, movement, and adhesion [28]. It also plays a significant role in cell signal transduction, transmitting signals to the nucleus [29,30]. As a membrane cytoskeleton connector, Ezrin interacts with multiple signaling pathways [31]. Studies by Orian-Rousseau et al. demonstrated that CD44v6 and Ezrin activate the ERK signal transduction pathway through their interaction, indicating a connection between Ezrin and this pathway [32]. This suggests that the Ezrin protein may be involved in various cell activities, including proliferation, migration, and apoptosis, through the ERK and Akt signaling pathways [33–35]. Ezrin is closely associated with molecules that regulate PI3K, AKT, Erk1/2, MAPK, and Rho pathways, which are crucial in controlling cell survival and migration signals [36]. Numerous studies indicate that abnormal Ezrin protein expression can enhance the metastatic capabilities of tumor cells and impact various aspects of tumor metastasis, potentially serving as a determinant factor [37–39]. In our study, we observed a significant correlation between Ezrin and adverse clinical features, as well as its

involvement in activating cancer-related signal pathways such as p53, cell cycle, NOTCH, and VEGF.

Furthermore, our research revealed that Ezrin is not only involved in various immune-related pathways such as antigen processing and immune cell activation but also plays a crucial role in tumor immune microenvironment (TIME) [40,41]. The TIME has been increasingly recognized as an influential factor in tumor growth and metastasis. The unique cellular and molecular characteristics of TIME can impact cancer progression by altering the balance between immune suppression and cytotoxicity within the tumor [42]. We observed a correlation between increased expression of Ezrin and elevated neutrophil presence. Numerous studies have also demonstrated the significant contribution of tumor-associated neutrophils to cancer initiation, development, and progression. Neutrophils possess diverse mechanisms through which they can sustain tumor growth, including impeding T cell activation, promoting genetic instability, and facilitating tumor cell proliferation, angiogenesis, and metastasis [43,44]. Hence, our findings indicate that Ezrin not only affects disease progression via a singular carcinogenic pathway but also interacts with the immune microenvironment to influence tumor development.

Immunotherapy has shown effectiveness in treating gastric cancer [45,46]. Researchers have proposed combining new molecules with immunotherapy to enhance its efficacy. However, so far, immune drugs with significant impact on gastric cancer patients have not been discovered. Currently, immunotherapy primarily relies on cytotoxic immune cells, monoclonal antibodies, and gene transfer vaccines [47]. The use of immune checkpoint inhibitors (ICIs) in particular is increasing rapidly [48–51]. Targeted immunotherapy that targets the programmed cell death 1 (PD-1) and programmed death ligand 1 (PD-L1) pathways has been a breakthrough in managing solid tumors [52–55]. Our study found that elevated levels of Ezrin, a protein, significantly increase the activation of various immune cell signaling pathways, including dendritic cells and natural killer cells. Furthermore, we observed that patients with high Ezrin expression were more responsive to PD-1 and CTLA-4 treatments. Therefore, our findings suggest that patients with high Ezrin expression may have a favorable immune environment that could benefit from immunotherapy.

In the broader context of gastric cancer progression, our findings on Ezrin's role are further illuminated when viewed through the lens of non-apoptotic cell death mechanisms. The diverse pathways of non-apoptotic cell death, including necroptosis, ferroptosis, and pyroptosis, have been increasingly recognized for their distinct roles in shaping the tumor microenvironment, influencing immune responses, and determining therapeutic outcomes. The interplay between Ezrin expression and these non-apoptotic pathways offers a deeper layer of complexity. For instance, elevated Ezrin expression might modulate the balance between apoptotic and non-apoptotic cell death, potentially favoring one pathway over the other under specific conditions. This balance can have profound implications for disease progression, metastatic potential, and therapeutic resistance.

Despite its contributions, this study has several limitations. Primarily, the sample size for the single-center research is restricted. Additionally, the correlation analysis heavily relies on online public databases, warranting the need for external validation using clinical datasets for enhanced reliability.

5. Conclusion

In this study, we used proteomics, bioinformatics, clinical sampling, and animal experiments to explore the significance of the protein Ezrin in gastric cancer. We found that Ezrin is closely related to the tumor microenvironment in gastric cancer. The protein also shows potential as a target in immunotherapy and chemotherapy and may have potential in clinical application. These findings contribute to the ultimate goal of providing more accurate and personalized diagnosis and treatment of cancer.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

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Ethics statement

The animal experiment was carried out at the Animal Experimental Center of the Key Laboratory of Myocardial Ischemia of the Second Afliated Hospital of Harbin Medical University in strict accordance with the scheme approved by the Animal Care and Use Committee (approval no. SYDW2021-072).

CRediT authorship contribution statement

Yanli Zhu: Writing – original draft, Data curation, Conceptualization. **Xue Zhang:** Software, Data curation. **Yi Chen:** Formal analysis. **Qianli Liu:** Methodology. **Jin Yang:** Software. **Xiaoxiao Fan:** Resources. **Hanjun Song:** Validation, Software. **Zhuoxin Cheng:** Writing – original draft, Conceptualization. **Shuang Liu:** Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e27155.

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