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Identification of ESM1 as a potential biomarker involving drug sensitivity and the tumor immune microenvironment that promotes proliferation of melanoma

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ABSTRACT

ESM1 may play a role in human cancers, but its role in melanoma remains unclear. This study investigated ESM1 expression in Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) databases and confirmed it through immunohistochemistry (IHC) and Western blotting (WB). Using the ESM1 gene expression levels, we divided TCGA samples into high and low-expression groups and identified differentially expressed genes (DEGs) between them. We then performed GO and KEGG enrichment analyses on these DEGs and explored the immune landscape while identifying anti-tumor drugs. ESM1 was found to be highly expressed in metastatic melanoma compared to primary melanoma and normal tissues. This was associated with increased numbers of immune-related cells and genes, as well as the activation of tumor progression pathways such as Notch and Wnt. In the high ESM1 expression group, the number of multiple immune-related cells and the expression of immune-related genes correlated with the presentation of ESM1, as well as the agonism of pathways related to tumor progressions. Immunohistochemistry and WB demonstrated a significant increase in ESM1 expression in metastatic lesions. Multiple GEO datasets showed higher ESM1 mRNA expression in malignant melanoma than in other benign tumors. ESM1 knockdown in a mouse model reduced tumor volume and weight related to the Wnt/-catenin and NOTCH signaling pathways. So, ESM1 is a promising biomarker for drug sensitivity, the tumor immune microenvironment, and the proliferation of cutaneous melanoma.

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Introduction

The annual incidence of skin cancer is greater than that of all other forms of cancer combined, and among skin cancers, melanoma (MM) is the deadliest because of its very rapid growth rate (1). The individual risk of developing MM depends on risk factors such as genetic mutations, susceptibility to MM, polymorphisms in genes or secondary risk regulator genes, and the degree of acute or chronic sun exposure (2). Each year, more than 320,000 people worldwide are diagnosed with MM, accounting for 1.7% of global cancer diagnoses, and nearly 60,000 deaths from MM. Over the past decades, the incidence of MM has increased in developed, predominantly fair-skinned countries (3). MM can be treated surgically at an early localized stage with good outcomes, and a 5-year relative survival rate of up to 98% (4). However, when a patient is diagnosed with advanced or metastatic cancer, the percentage of patient survival decreases dramatically because treatment options are very limited in the later stages of the tumor (5).

Endothelial cell-specific molecule 1 (ESM1), an endocan located on chromosome 5q11.2, was initially shown to play a key role in the regulation of endothelial cell function (6). Several recent studies have suggested that ESM1 may also be involved in the development, progression, and prognosis of some human cancers, such as head and neck squamous cell carcinoma, hepatocellular carcinoma, and bladder cancer (7–9). Much new evidence now demonstrates that ESM1 expression is elevated in a wide range of cancers. It is also noteworthy that ESM1 expression levels are closely associated with tumor aggressiveness and tumor vascularization (10,11). Furthermore, ESM1 has been found to be involved in multiple aspects of tumorigenesis, such as promoting normal cell carcinogenesis, regulating cell proliferation and survival cycles, and regulating the migration and invasion of tumor cells. In addition, ESM1 is closely associated with tumor drug resistance (12). ESM1 had an impact on clinical outcomes after chemotherapy in patients with advanced gastric cancer, and its overexpression was strongly associated with radiotherapy resistance in breast cancer, suggesting a potential clinical value of ESM1 in cancer patients (13,14). It has been demonstrated that overexpression of ESM1 can cause tumor cell formation in the nontumorigenic epithelium, and overexpression of ESM1 in tumorigenic cells can significantly increase the growth rate of tumors (15). However, the level of expression and clinical significance of ESM1 in most cancers remain elusive, and no studies have reported its relevance in MM. Therefore, further investigation is warranted to identify the gene function of ESM1 in MM development.

In this study, we analysed the immune cell infiltration of ESM1 in metastatic MM and its relationship with related signalling pathways and genes to confirm the regulatory role of ESM1 in the metastasis of MM. Furthermore, we performed a drug sensitivity analysis to find effective drugs for the clinical treatment of MM and improving pa-

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tient survival. To achieve this, we used MM patient specimens and two MM cell lines to conduct a combined study, aiming to reveal the possibility of ESM1 becoming a therapeutic target for MM patients.

Materials and Methods

Data retrieval and preprocessing

We obtained all RNA-seq data and clinical information regarding MM from the TCGA (https://tcga-data.nci.nih.gov/tcga) and GEO (https://www.ncbi.nlm.nih.gov/geo/) databases. The analysis included four datasets, namely TCGA-MM, GSE12391, GSE46517, and GSE112509.

Within the GSE12391 chip data, there were common melanocytic nevi (CMN) (n = 18), dysplastic nevi (DN) (n = 11), primary radial growth phase malignant melanomas (RGPM) (n=8), primary vertical growth phase malignant melanomas (VGPM) (n=15) and melanoma metastases (MTS) (n=5). Meanwhile, the GSE46517 microarray data set contained CMN (n = 9), primary melanoma (PM) (n = 31), and metastatic melanoma (MM) (n = 73). Finally, GSE112509 sequencing data included CMN (n = 23) and PM (n = 57). Subsequently, we extracted the expression matrix of ESM1, along with the corresponding clinical data, for further analysis.

The ESM1 gene analysis

For this study, the Tumor Immune Estimation Resource (TIMER) database (https://cistrome.shinyapps.io/timer/) was used to examine ESM1 mRNA expression in different types of cancer. The statistical analyses were performed using R software v4.1.3. The "limma" R package was employed to illustrate how ESM1 mRNA was distributed in primary and metastatic MM tissues. A correlation analysis based on the Pearson algorithm was undertaken to identify co-expressed genes in ESM1. Heatmaps were utilized to indicate the distribution of various clinical factors in high and low ESM1 expression groups. Finally, the "limma" package in R was used to recognize the differentially expressed genes (DEGs) between the high and low-expression ESM1 groups (FDR>0.05 and |logFC|>1.5). Furthermore, the "clusterprofiler" package in R was implemented to perform Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis on DEGs, examining these DEG-related enrichment pathways and gene functions in more depth.

Gene Set Enrichment Analysis and Protein-Protein Interaction networks

The gene set "2.cp.kegg.v7.5.1.symbols.gmt" was obtained from the Molecular Signatures Database (MSigDB, https://www.gsea-msigdb.org/gsea/msigdb/). In this study, Gene Set Enrichment Analysis (GSEA) was utilized to investigate the relevant roles of the Notch and Wnt signaling pathways. The Protein-Protein Interaction (PPI) network was constructed using the Search Tool for the Retrieval of Interacting Genes database (STRING, http://www.stringdb.org) to predict the protein-protein interactions of differentially expressed genes (DEGs) in the Notch and Wnt signaling pathways. Only genes with an interaction score > 0.15 were retained, and Cytoscape software v3.9.1 was used to visualize the PPI network.

Exploration of the immune landscape and identification of antitumor drugs

The R packages "CIBERSORT" and "ESTIMATE" were utilized to obtain pertinent data regarding tumor purity, immune score, and ESTIMAT score, as well as to compute the content and infiltration density of immune cells in the high and low ESM1 expression groups. In order to further investigate the relationship between the ESM1 gene and the level of immune infiltration, CIBERSORT was used to calculate the level of infiltration of 22 immune cells. A co-expression analysis was also performed on the expressions of the ESM1 gene and genes related to immune checkpoints. Finally, to evaluate the response to therapy and explore common antitumor drugs for MM treatment, the R package pRRophetic was used to calculate the half inhibitory concentration (IC50) of drugs and compare this metric between different groups.

Immunohistochemistry staining and scoring

Primary skin specimens and metastatic lymph node specimens were obtained from the Dermatopathology Department of the Affiliated Hospital of Qingdao University, China, from June 2018 to March 2020. None of the patients had received any chemotherapy, targeted therapy, or immunotherapy before the operation. Finally, this study included seven cases of MM metastasis and in situ tissues. The study was approved by the ethical committee of the Affiliated Hospital of Qingdao University (20190125). Immunohistochemistry staining was performed as described elsewhere, and a specific antibody was used, namely ESM1 (Abcam, ab224591). The ESM1-positive cells were counted in five random high-power fields (×400), and the mean positive cell ratio was calculated. Then, a combined score of ESM1 was conducted to evaluate the intensity and distribution of ESM1 cytoplasmic and membrane staining. The ESM1-positive cells with cytoplasmic staining were assigned a score of 0 (no discernible ESM1 staining), 1 (1%-49%), 2 (50%-79%), or 3 (≥80%). The ESM1-positive cells with membrane staining were graded as follows: a score of 0 (absent), 1 (1%-19%), 2 (20%–49%), or 3 (≥50%). The two scores were summed to yield an overall score ranging from 0 to 6, denoting the expression level of ESM1. The IHC staining was independently scored by three authors in a blinded manner. For any score difference among observers, the consistency could be evaluated using a multihead microscope, and then the final score could be recorded.

Western blot analysis

The total protein was extracted from cellular lysates and analysed via Western blot analysis, in accordance with previously established methods. The Cell Lysis Buffer provided by Beyotime Company (P0013) was utilized as the reagent for total protein extraction, and Beyotime's Nuclear and Cytoplasmic Protein Extraction Kit (P0027) was employed. The following antibodies were utilized in Western blotting: anti-ESM1 antibody (ab103590), anti-β-catenin antibody (ab32572), anti-C-Myc antibody (ab32072), anti-DLL4 antibody (Abcam, ab7280), anti-Notch4-ICD antibody (Abcam, ab33163), and anti-GAPDH antibody (Sigma, G9545). The relative expression of proteins was normalized to GAPDH.

Cell lines and lentiviral vector transfection for silencing ESM1 expression

The metastatic melanoma cell lines SK-MEL-1/SK-MEL-5 (ATCC1 HTB-67 ™/HTB-70 ™) were acquired from the American Type Culture Collection and preserved in our laboratory. The cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, CA, USA) supplemented with 10% fetal calf serum. To silence ESM1 expression in SK-MEL-1/SK-MEL-5 cell lines, a lentiviral vector targeting ESM1 was constructed using the shRNAi vector pGMLV (pGMLV-GFP-vshRNA-ESM1, Genomeditech, Shanghai, China). The shRNA-ESM1 lentiviral vector was generated by incorporating the following shRNA-targeting oligo sequence for ESM1:

Top Strand: 5'-CACCGGCCGCCTGGAGCAATAATTACGAATAATTATTGCTCCAGGCGGCC-3'

Bottom Strand: 5'-AAAAGGCCGCCTGGAGCAATAATTATTCGTAATTATTGCTCCAGGCGGCC-3'

The recombinant pGMLV-GFP-vshRNA-ESM1 construct was confirmed through PCR and DNA sequencing. Lentivirus packaging was conducted in 293T cells, followed by transfection of shRNA-ESM1 into SK-MEL-1/SK-MEL-5 cells. A mock lentiviral vector was used as a negative control. The most effective shRNA fragment against ESM1 was determined through real-time PCR analysis.

Mouse xenograft study

We procured all male BALB/c nude mice aged between 4-6 weeks from the Shanghai SLAC Laboratory Animal Co. Ltd. and maintained them under standard pathogen-free (SPF) conditions at the Experimental Animal Center of the Affiliated Hospital of Qingdao University. Our study was conducted after obtaining the necessary approvals from the Institutional Animal Care and Ethics Committee of the Affiliated Hospital of Qingdao University. For the tumor formation assay, we randomly allocated 24 BALB/c nude mice aged between 4-6 weeks into four groups, and we subcutaneously injected SK-MEL-1/ESM1-shRNA cells, SK-MEL-1/NC-shRNA cells, SK-MEL-5/ESM1-shRNA cells, and SK-MEL-5/NC-shRNA cells at a dose of 2×10^7 cells per animal for each group in the armpit. We recorded the size of the primary tumor every other day using a calliper, and only measurable tumors were included in the mean tumor volume calculation for each tumor cell clone at each time point. We humanely euthanized all animals using carbon dioxide inhalation 32 days after injecting the cells because the largest tumors reached approximately 15 mm in diameter. We extracted proteins from all transplanted tumors for the subsequent WB experiments.

Results

Expression of ESM1 mRNA in MM

The present study aimed to explore the expression of ESM1 mRNA in MM and its relationship to clinical features. To accomplish this objective, RNA-Seq data and clinical information on MM patients were acquired from the TCGA databases. Initial analysis was performed on the TCGA-MM dataset for validation, from which the expression matrix of ESM1 and the corresponding clinical data were retrieved. In order to determine the differences in ESM1 mRNA expression between tumour and adjacent normal tissues, ESM1 mRNA expression levels across various cancers were analyzed using the TIMER2.0 database. The results indicated that ESM1 was highly expressed in the majority of cancer tissues when compared with adjacent normal tissues (Figure 1A). Notably, metastatic MM showed higher ESM1 expression than in situ tissues. TCGA data revealed that ESM1 mRNA expression was lower in situ than in metastatic tissues (Figure 1B). To identify the genes co-expressed with ESM1, correlation analysis using the Pearson algorithm was performed (Figure 1C; Supplementary Table 1). Heatmaps were utilized to represent the distribution of different clinical features in high and low-expression ESM1 groups (Figure 1D). Based on the heatmaps, all samples were classified into high and low-expression ESM1 groups.

Identification of Differentially Expressed Genes

The "limma" package in R was used to identify DEGs between the high and low expression ESM1 groups (FDR > 0.05 and |logFC| > 1.5) (Figure 2A). To further investigate the pathways and functions associated with these DEGs, we conducted GO and KEGG enrichment analyses to evaluate the potential molecular mechanisms of ESM1. In the GO enrichment analysis of differentially expressed MRGs, the biological process (BP) terms were correlated with the regulation of synapse structure or activity (GO:0050803), and synapse organization (GO:0050808); cellular component (CC) terms were related to the synaptic membrane (GO:0097060) and collagen-containing extracellular matrix (GO:0062063); and molecular function (MF) terms were associated with receptor ligand activity (GO:0048018) and signaling receptor activator activity

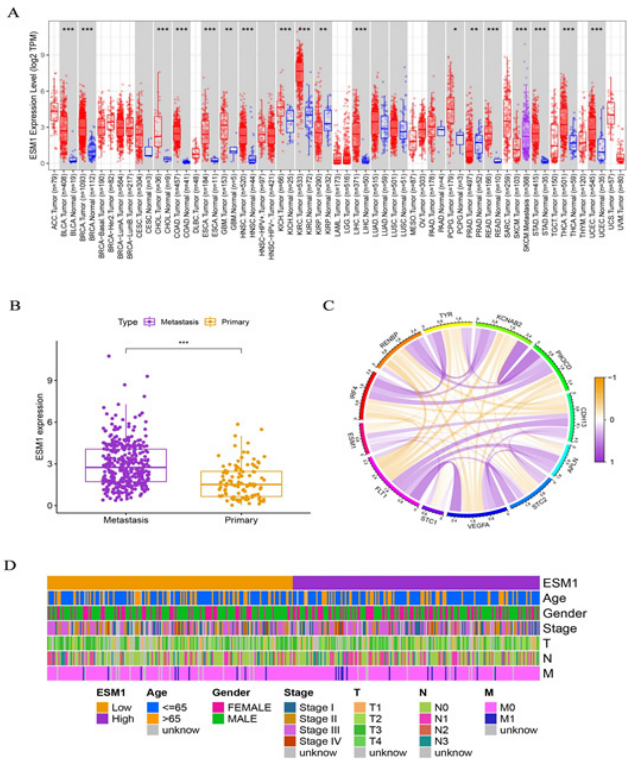

Figure 1. (a) ESM1 expression levels in different cancer types from TCGA data in TIMER. (b) ESM1 mRNA expression in primary and metastatic MM tissues. (c) Correlation analysis of ESM1 and its 11 coexpressed genes. (d) Heatmaps of the distribution of different clinical features in the high and low-expression ESM1 groups.

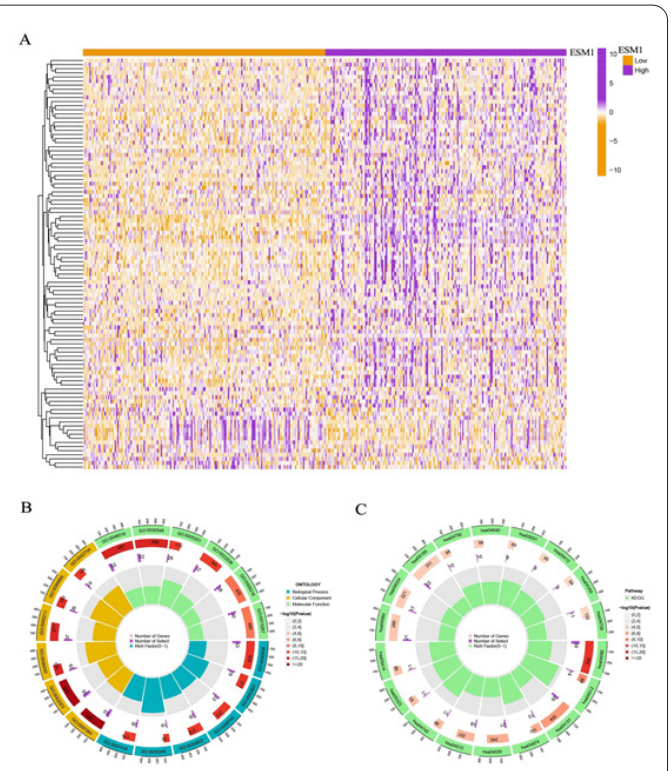

Figure 2. (a) DEGs between high and low expression ESM1 groups. (b) GO analysis of biological processes, cellular components and molecular functions of ESM1. (c) KEGG analysis of the correlation between the ESM1 gene and some related pathways.

(GO:0030546) (Figure 2B; Supplementary Table 2). In addition, we performed a KEGG enrichment analysis, which highlighted the correlations of 18 pathways (Figure 2C and Supplementary Table 3).

Gene set Enrichment Analysis and Protein-Protein Interaction networks

The gene set named "2.cp.kegg.v7.5.1.symbols.gmt" was acquired from the Molecular Signatures Database. To investigate the significance of the Notch and Wnt signaling pathways in the study, GSEA was utilized. The results of the analysis suggested that the Notch and Wnt signaling pathways were universally activated during the process of ESM1 expression (refer to Figure 3A).

To reveal the protein-protein interactions of DEGs in the Notch and Wnt signaling pathways, a PPI network was constructed using the Search Tool for the Retrieval of Interacting Genes database. Subsequently, genes with an interaction score greater than 0.15 (including 25 Wnt-related genes and 7 Notch-related genes) were selected and utilized to construct the PPI networks, illustrated in Figure 3(A, B) using Cytoscape software, version 3.6.0.

Exploring the immune landscape and identifying antitumor drugs

Initially, we obtained the stromal score, immune score, and ESTIMATE score for tumors from relevant databases. We computed the content and infiltration density of immune cells in both high and low ESM1 expression groups to reveal the relationship between the ESM1 gene and immune infiltration levels (Figure 4A). CIBERSORT was employed to calculate the level of infiltration of 22 immune cells (Figure 4B), indicating that immune infiltration levels were higher in the low ESM1 expression group. The relationship between infiltrating immune cells and

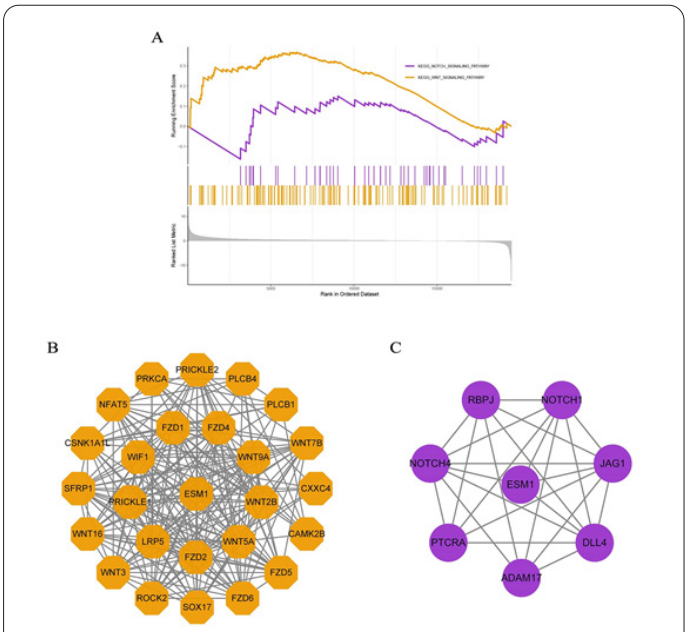

Figure 3. (a) KEGG of Notch and Wnt signaling pathways. (b) PPI of DEGs in the Notch and Wnt signaling pathways. (c) PPI of DEGs with an interaction score > 0.15.

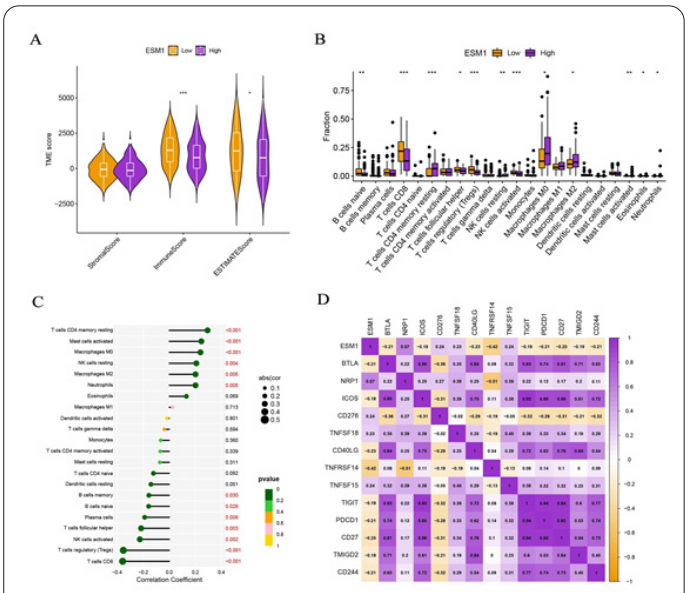

Figure 4. (a) Stromal score, immune score and ESTIMAT score of tumors. (b) Immune infiltration of high and low expression ESM1 groups. (c) Relationship of infiltrating immune cells with the ESM1 gene. (d) Some immune checkpoint-related genes and the ESM1 gene and their correlation of expression.

ESM1 expression is presented in Figure 4C. Further, we conducted a co-expression analysis of the ESM1 gene and immune checkpoint-related genes. The outcomes demonstrated that almost all 13 immune-related genes showed a negative correlation with ESM1 expression (Figure 4D).

To evaluate therapy response and explore common antitumor drugs for Multiple Myeloma treatment in the clinical setting, we used the R package pRRophetic to calculate the IC50 of drugs and compared them between different groups. We found a significant difference in the IC50 of 32 commonly available Antitumor drugs used for MM treatment in different groups (Figure 5).

Validation of ESM1 by in-house data

In order to further validate our findings, we analysed the expression levels of the ESM1 gene in three dif-

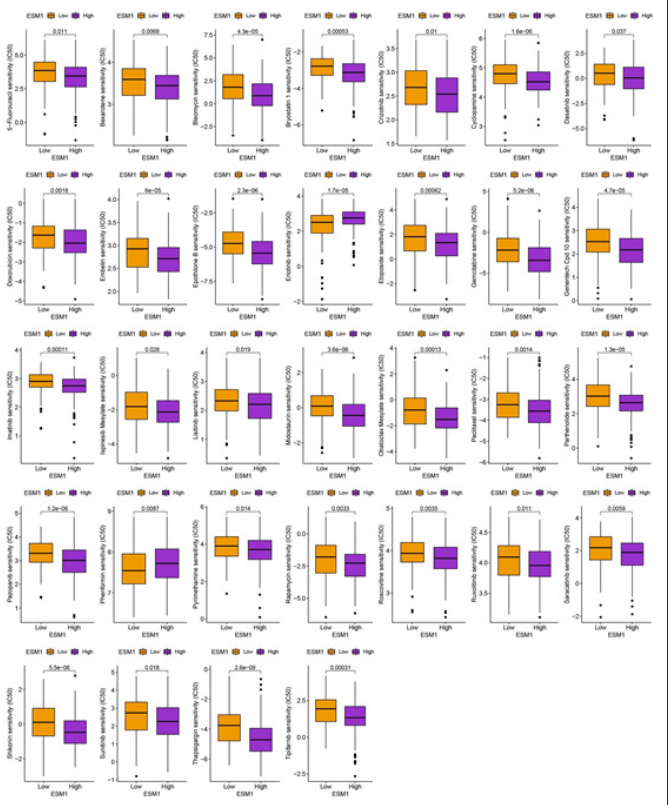

Figure 5. Identification of commonly used antitumor drugs.

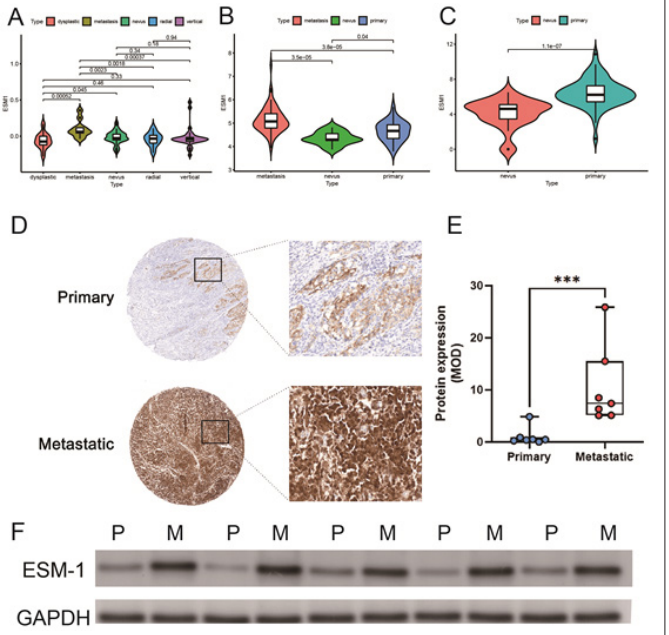

Figure 6. (a-c) Expression levels of the ESM1 gene in the GSE12391, GSE46517, and GSE112509 datasets. (d, e) ESM1 has a higher expression level in metastatic MM tissues than in primary MM. IHC was used to validate the significant difference in ESM1 expression between seven pairs of primary and metastatic MM samples. (f) WB to test the difference in ESM1 between primary and metastatic MM samples.

ferent datasets. Figures 6A, 6B and 6C demonstrate the expression levels of ESM1 in GSE12391, GSE46517 and GSE112509, respectively. We found that ESM1 gene expression was significantly higher in metastatic melanoma than in primary lesions, pigmented nevi and dysplastic nevi. ESM1 gene expression was also significantly higher in melanoma than in nevi. To validate this significant difference in ESM1 expression, an IHC test was conducted on 7 pairs of primary and metastatic MM samples, as shown in Figures 6D and 6E. The staining intensity of ESM1 was notably higher in metastatic lesions than in primary lesions. Finally, to confirm this difference, we used WB to test the expression of ESM1 in primary and metastatic MM samples. As shown in Figure 6F, the expression of the ESM1 protein was significantly higher in metastatic tissue than in primary tissue.

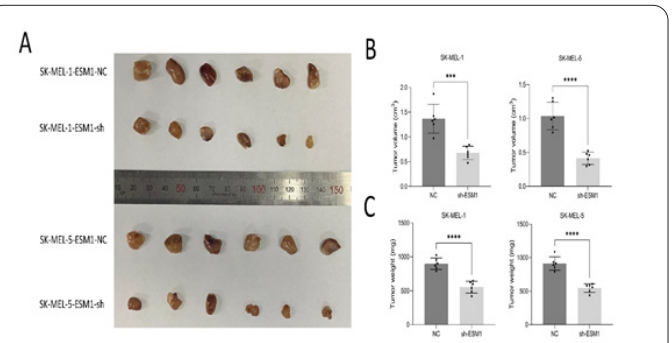

Figure 7. ESM1 promotes tumorigenesis in mice. (a, b, c) Xenograft weight (mg) and size (cm) were measured.

ESM1 enhances tumorigenicity in mice

To validate the function of endogenous ESM1 in vivo, we stably transfected SK-MEL-1/5 cells and subcutaneously injected them into BALB/c-nu mice. Weekly tumor growth was measured in the xenograft mouse model (Figure 7). Results in Figure 7 show that ESM1 knockdown markedly inhibited tumor growth in comparison to the control. We also examined xenografts for the expression of ESM1, Notch-ICD, DLL4, C-Myc, and β-catenin through the WB.

Results in Figure 8 indicate that ESM1 knockdown impaired Notch4-ICD, DLL4, and β-catenin expression in the nucleus, but total β-catenin expression remained unaffected. These findings suggest that ESM1 contributes to tumorigenesis in vivo and may activate the Wnt/β-catenin and Notch signaling pathways.

Drawing from in vivo experiments with human MM cell lines and literature, we have depicted in Figure 9 that Wnt/β-catenin and the Notch pathway may be involved in

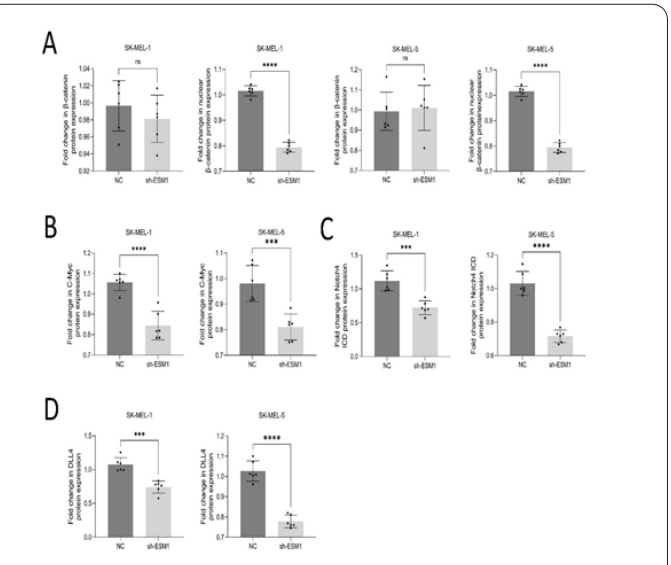

Figure 8. WB analysis of the expression of β-catenin, C-Myc, Notch4-ICD and DLL4 in sh-ESM1/scramble xenograft tumors.

ESM1-driven tumorigenesis.

Discussion

Although primary MM is better treated in its early stages, the five-year survival rate remains low for metastatic and late-stage MM (16). ESM1, also known as endothelial cell-specific molecule-1, is an endothelium-associated proteoglycan that may be involved in neoangiogenesis and has been extensively studied in recent years as a promising biomarker for endothelial dysfunction, inflammation, and neoplasia (17,18). Furthermore, it was found that the expression of ESM1 genes plays a crucial role in tumor migration and metastasis, and inhibition of this gene expression can effectively control tumor progression. In our study, we explored the expression of ESM1 across cancers and showed that the degree of ESM1 expression was higher in a variety of common tumor tissues than in normal tissues. More notably, ESM1 gene expression was significantly higher in metastatic MM than in primary MM, indicating that the impact of this gene on MM cannot be ignored.

The WNT signaling pathway controls important cellular processes throughout embryonic development and adulthood, and therefore, any laxity in this signaling may lead to a wide range of pathologies, including various cancers (19,20). In addition, it is important for maintaining genetic stability, regulating cell fate and differentiation, cell proliferation, cell viability, apoptosis, and stem cell maintenance. In addition, a recent review summarized the roles of a wide range of modifications of the Wnt signaling pathway that affect the development of various tissues and organs, generate relevant genetic phenotypes, sustain tumor formation, and promote tumor invasion (21–24). In this study, we performed GSEA on the high ESM1 expression group, and the results showed that the activation of the Notch and Wnt signaling pathways was greatly increased in the tumors of this group, indicating that the Notch and Wnt signaling pathways are closely related to the expression of the ESM1 gene and play a role in promoting the metastasis and invasion of MM. β-Catenin has been shown to promote tumor recurrence and reduce patient survival. A study showed that although tumors with β-catenin mutations tend to have a low-risk profile, they are strongly associated with a poorer prognosis for the tumor, with a significant increase in the rate of disease recurrence, leading to a substantial reduction in overall survival (25). Another study also showed that mutation and activation of β-catenin lead to a much lower survival rate in patients with endometrial cancer (26). However, the exact role of WNT signaling in melanoma initiation and progression remains highly controversial to date. extensive studies. In this study, knockdown of ESM1 significantly reduced the nuclear expression of β-catenin and the expression of C-Myc, a downstream effector molecule of WNT signaling, which confirmed that ESM1 was involved in the activation of downstream effector molecules by assisting β-catenin in entering the nucleus (27).

Notch was first identified as an oncogene in T-ALL, and alterations in Notch receptors were discovered in various cancers (28,29). The activation of the Notch pathway in breast cancer, lung adenocarcinoma, hepatocellular cancer, and colorectal cancer was determined to be oncogenic (30,31). In addition, an in vitro experiment showed that

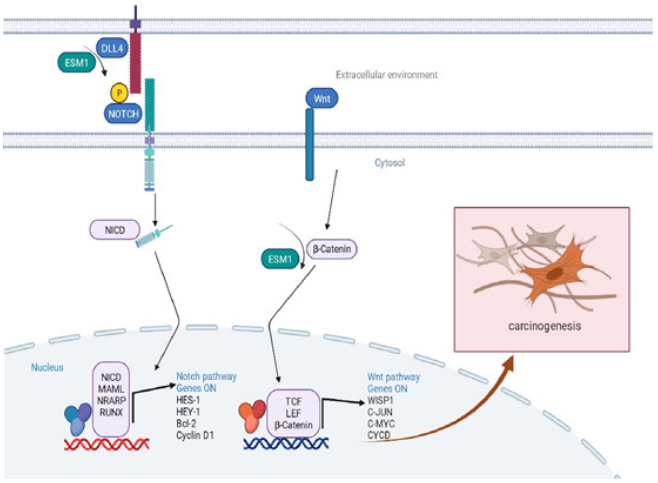

Figure 9. Involvement of Wnt/β-catenin and Notch pathways in ESM1-driven tumorigenesis.

ESM1 enhanced CDK1- and p21-mediated mitosis in the G2/M phase transition and that cells proliferated through the DLL4-Notch signaling pathway in the human adrenocortical tumor cell line SW13 (32,33). This provides new ideas for chemotherapy and immunotherapy of tumors in clinical practice. DLL4 is an important ligand of the Notch signaling pathway and assists Notch in promoting tumor invasion (34,35). A study showed that ESM1 can interact closely with DLL4 and is involved in the Notch signaling pathway during adrenocortical carcinogenesis and progression (36). According to our analysis, ESM1 is closely related to and active in DLL4. DLL4 is an important ligand of the Notch signaling pathway. Therefore, ESM1 may interact closely with DLL4 and be involved in the Notch signaling pathway during MM development and progression. Mechanistically, this may be related to the fact that ESM1, as a secreted protein, binds to DLL4 outside the cell and activates Notch signaling. This result was confirmed by the interaction between the ESM1 homologous C. elegans proteins OSM-11 and LIN-12 (37).

In the comparative analysis of immune infiltration in the high and low ESM1 expression groups, we found that the infiltration levels of activated natural killer (NK) cells, regulatory T cells, CD^{8+} T cells, and naive B cells were lower, while the infiltration levels of M0 macrophages, resting NK cells, and M2 macrophages were higher in the high ESM1 expression group. NK cells are powerful effectors of innate immunity and constitute the first line of defense against cancer. Their ability to eliminate transformed cells and preserve normal healthy cells plays a particularly important role in preventing cancer metastasis (38). It has also been shown that NK cell infiltration within melanoma tissue is associated with improved overall survival in solid cancers, revealing the importance of NK cells in melanoma (39). CD^{8+} T cells activated by cancer immunotherapy induce cell death via the perforin granzyme and Fas-Fas ligand pathways, resulting in tumor clearance (40). Thus, it is evident that high expression of ESM1 negatively affects both immune infiltration and the immunotherapeutic response in MM patients, which may increase the difficulty of treatment in MM patients. The statistically significant differences in the IC50 of 32 commonly used antitumor drugs used for MM treatment in different ESM1 expression groups suggest that we can target different antitumor

drugs for different ESM1 expression levels. The aim of this study was to verify that the ESM1 gene has a promoter role in MM invasion and metastasis and is closely linked to signaling pathways and related genes associated with tumor development, which together lead to tumor metastasis. In addition, we performed immunohistochemical analysis and established a xenograft model to further test our hypothesis. We believe that the results of this study are reliable.

However, we are also aware that this study has several flaws and limitations. Since the molecular mechanisms underlying the role of the ESM1 gene in MM are not fully understood and relevant in vivo experiments are not sufficient, it would be wise to validate it through further basic experiments, such as CO-IP experiments, to validate the interacting protein of ESM1. In addition, an expanded sample size is needed to validate this finding. In addition, we will validate the accuracy of this study with reasonable in vivo and in vitro experiments in future studies.

In conclusion, our study reveals the effect of ESM1 on the metastatic nature of MM and provides new insights into the pharmacological treatment of MM, which can help to clinically improve the survival rate of patients with advanced or metastatic MM.

Acknowledgments
Not applicable.

Interest conflict
The authors declare no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

Author's contribution
Design of the study: Miaomiao Wang, Guanzhi Chen; Collection of the tissue specimen and analysis of clinicopathologic data: Miaomiao Wang, Hongfen Ge, Shuya Liang; In vivo experiments and data analysis: Miaomiao Wang, Shuya Liang, Hongfen Ge. Drafting and revision of the manuscript: Miaomiao Wang, Guanzhi Chen. All authors read and approved the final version of the manuscript.

Supplementary Table S1. ESM1 coexpressed genes.
Supplementary Table S2. GO enrichment analysis.
Supplementary Table S3. KEGG enrichment analysis.

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